FLOWSIGHT ®

INSPIRE for FlowSight Software
User's Manual

September 2013
FlowSight version 100.2.195

Log in to your account at www.amnis.com/login.html to check for updates.

Preface

Patents and Trademarks

Amnis Corporation's technologies and products are protected under one or more of the following U.S. patents: 6211955; 6249341; 6256096; 6473176; 6507391; 6532061; 6563583; 6580504; 6583865; 6608680; 6608682; 6618140; 6671044; 6707551; 6763149; 6778263; 6875973; 6906792; 6934408; 6947128; 6947136; 6975400; 7006710; 7009651; 7057732; 7079708; 7087877; 7190832; 7221457; 7286719; 7315357; 7450229; 7522758, 7567695.

Additional U.S. and corresponding foreign patent applications are pending.

Amnis, the Amnis logo, ImageStream, FlowSight, INSPIRE, IDEAS, SpeedBead, FISHIS are registered or pending U.S. trademarks of Merck KGaA.

All other trademarks are acknowledged.

Disclaimers

The screen shots presented in this manual may vary in appearance from those on your computer, depending on your display settings.

The Amnis® FlowSight® cell analysis system is for research use only and not for use in diagnostic procedures.

Technical Assistance

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Chapter 1: General Information and Safety

This section covers safety information for operating the Amnis FlowSight® flow cytometer. Anyone who operates the FlowSight should be familiar with this safety information. Keep this information readily available for all users.

The FlowSight imaging flow cytometer is manufactured by Amnis Corporation and has a rated voltage of 100-240 VAC, a rated frequency of 50/60 Hz, and a rated current of 1.5 A. The years of construction were 2011-2013 and the product contains CE Marking.

Environmental conditions: This instrument was designed for indoor use at an altitude of less than 2000 m; at a temperature from 5 through 25 ° C; and at a maximum relative humidity of 80%, non-condensing. During instrument operation the ambient temperature should be maintained within +/- 2° C. The mains power supply may not fluctuate more than +/- 10% and must meet transient over voltage category (II). The instrument is evaluated to Pollution Degree 2.

Noise level: The noise level of the FlowSight is less than 70 dB(A).

Weight: 56 kg.

Ventilation: Provide at least 3 inches of clearance behind the instrument to maintain proper ventilation.

Disconnection: To disconnect the instrument from the power supply, remove the plug from the socket outlet–which must be located in the vicinity of the machine and in view of the operator. Do not position the instrument so that disconnecting the power cord is difficult. To immediately turn the machine off (should the need arise), remove the plug from the socket outlet.

Transportation: The FlowSight relies on many delicate alignments for proper operation. The machine may be moved only by an Amnis representative.

Cleaning: Clean spills on the instrument with a mild detergent. Using gloves clean the sample portal and sample elevator with a 10% bleach solution. Dispose of waste using proper precautions and in accordance with local regulations.

Preventative maintenance: The FlowSight contains no serviceable parts. Only Amnis-trained technicians are allowed to align the laser beams or otherwise repair or maintain the instrument. The instrument fluidic system is automatically sterilized after each day's use. This reduces the occurrence of clogging. Tubing and valves are replaced by Amnis service personnel as part of a routine preventive maintenance schedule.

Access to moving parts: The movement of mechanical parts within the instrument can cause injury to fingers and hands. Access to moving parts under the hood of the FlowSight is intended only for Amnis service personnel.

Protection impairment: Using controls or making adjustments other than those specified in this manual can result in hazardous exposure to laser radiation, in exposure to biohazards, or in injury from the mechanical or electrical components.

FCC compliance: This equipment has been tested and found to comply with the limits for a Class A digital device, pursuant to part 15 of the FCC rules. These limits were designed to provide reasonable protection against harmful interference when the equipment is used in a commercial environment. This equipment generates, uses, and can radiate radio-frequency energy and, if not installed and used in accordance with the instruction manual, can cause harmful interference to radio communications. The operation of this equipment in a residential area is likely to cause harmful interference–in which case the user will be required to correct the interference at the user's own expense.

Declaration of Conformity

DECLARATION OF CONFORMITY

IN ACCORDANCE TO ISO/IEC GUIDE 22
FOR A
Cellular Analyzer

MANUFACTURER: Amnis Corporation
2505 Third Avenue, Suite 210
Seattle, WA 98121
Phone: 206.576.6865

MODEL NUMBER: FlowSight
REPORT #: AMNI0008 and F2LQ4537
DIRECTIVES: EMC Directive (2004/108/EC) & Low Voltage Directive (2006/95/EC)
STANDARDS:

- Electrical Equipment for Measurement, Control and Laboratory Use – EMC Requirements, Part 1: General Requirements, EN 61326-1:2006 edition
- Industrial, Scientific, and Medical Equipment – Radio-Frequency Disturbance Characteristics – Limits and Methods of Measurement, CISPR 11:2009 edition
- Information Technology Equipment – Radio Disturbance Characteristics – Limits and Methods of Measurement, EN 55022:1998 CISPR 22:1997 edition
- Electromagnetic Compatibility (EMC) – Part 3-2: Limits – Limits for Harmonic and Emissions (Equipment Input Current ≤16 A per phase), IEC 61000-3-2:2009 edition
- EMC - Part 3-3: Limits - Limitations of voltage changes, voltage fluctuations and flicker, in public low-voltage supply-systems, for equipment with rated current <=16 A per phase and not subject to conditional connections; IEC 61000-3-3:1995 edition
- Electromagnetic Compatibility – Part 4: Testing and Measurement Techniques – Section 2: Electrostatic Discharge Immunity Test, EN 61000-4-2:2008 edition
- Electromagnetic Compatibility – Part 4: Testing and Measurement Techniques – Section 3: Radiated, Radio-Frequency, Electromagnetic Field Immunity Test, EN 61000-4-3:2008 edition
- Electromagnetic Compatibility – Part 4: Testing and Measurement Techniques – Section 4: Electrical Fast Transient/Burst Immunity Test, EN 61000-4-4:2004 edition
- Electromagnetic Compatibility – Part 4: Testing and Measurement Techniques – Section 5: Surge Immunity Test, EN 61000-4-5:2005 edition
- Electromagnetic Compatibility – Part 4: Testing and Measurement Techniques – Section 6: Immunity to Conducted Disturbances, Induced by Radio-Frequency Fields, EN 61000-4-6:2008 edition
- Electromagnetic Compatibility – Part 4: Testing and Measurement Techniques – Section 8: Power Frequency Magnetic Field Immunity Test, EN 61000-4-8:2001 edition
- Electromagnetic Compatibility – Part 4: Testing and Measurement Techniques – Section 11: Voltage Dips, Short Interruptions, and Voltage Variations Immunity Test, EN 61000-4-11:2004 edition
- Safety Requirements for Electrical Equipment for Measurement, Control, and Laboratory Use Part 1: General Requirements, EN 61010-1:2001, 2nd edition

AUTHORIZED REPRESENTATVE:
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08015 Barcelona, Spain
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Contact: Jose Maria Alonso
+34 916 630 550

TEST FACILITY:
F-Squared Laboratories
26501 Ridge Road
Damascus, MD 20872 USA

Northwest EMC
22975 NW Evergreen Parkway, Ste 400
Hillsboro, OR 97124 USA

The Cellular Analyzer, Model FlowSight is in effective conformance to the Directives and Standards referenced above.

Authorized by: Richard Esposito

Date: 27 July 2011

Name: Richard Esposito

Title: Quality Assurance Officer

Explanation of symbols

Explanation of symbols

Label	Location	Hazard
	Waste tank	Risk of exposure to transmissible biological disease.
	Power supply cover	Risk of injury by electric shock.
	Power supply	Protective earth ground.
	Inside rear frame panel	Risk of exposure to hazardous laser radiation.
	interior, side panels near release mechanisms and next to hood interlocks	Risk of exposure to hazardous laser radiation.
	On the back of the instrument	No laser radiation is accessible to the user during normal instrument operation.

Electrical safety

Equipment ratings: The FlowSight is rated to the following specifications: 100-240 VAC, 50/60 Hz, and 1.5 A.

Electrical hazards are present in the system, particularly in the main power supply. To protect against electrical shock, you must connect the instrument to a properly grounded receptacle in accordance with the electrical code that is in force in your region.

Sécurité Electronique

Alimentation: 100-240 V altenatif, 50/60 Hz, 1.5 A.

Les hazards électrique se trouvent dans l'appareil, surtout prés de la source d'alimentation. Pour éviter les choks électriques, introduire la lame le plus large de la fiche dans la borne correspondante de la prise et pousser à fond.

Laser safety

The FlowSight is a Class 1 laser device and complies with the U.S. FDA Center for Devices and Radiological Health 21 CFR Chapter 1, Subchapter J. No laser radiation is accessible to the user during normal instrument operation. When the hood is removed , interlocks on the instrument turn the lasers off.

The FlowSight may have the following lasers:

Table 2

Wavelength	Maximum Power
400-413 nm	150 mW
483-493 nm	60 mW
558-562 nm	50 mW
635-647 nm	150 mW
775-795 nm	90 mW

The following laser warning label appears on the interior side panels near release mechanisms.

Caution: Using controls, making adjustments, or performing procedures other than those specified in this manual may result in hazardous exposure to laser radiation.

Sécurité Laser

L'FlowSight c'est une appareil au laser, Classe I, qui se conforme à U.S. FDA Center for Devices and Radiological Health 21 CFR Chapitre 1, subchapitre J. Aucune radiations laser sont accessible a l'utilsateur pendant le fonctionnement normal. Quand le capot est ouvert, les enclenchements eteindents les lasers.

FlowSight peut avoir les lasers suivants:

Table 3

Longueur d'opnde	La Puissance Maximale
400-413 nm	150 mW
483-493 nm	60 mW
558-562 nm	50 mW
635-647 nm	150 mW
775-795 nm	90 mW

Les etiquettes d'avertissement suivantes sont placeés dans l'interior

Les etiquettes d'avertissement suivantes sont placeés dans L'Intérieur, de panneaux latéraux pr s de mécanismes de libération.

Avertissement: L'utilisation des commandes ou les rendement des procedures autres que celle preciseés aux presentes peuvent provoquer une radioexposition dangereuse.

Biological safety

Biohazards: The FlowSight is rated at BSL1. Do not load or flush samples containing infectious agents without first exposing the sample to inactivating conditions. It is recommended that samples be fixed in 2% paraformaldehyde for at least 10 minutes before running the samples on the FlowSight.

The use, containment and disposal of biologically hazardous materials are required to be in accordance with Personnel Protective Equipment Directive 93/95/E and are the responsibility of the end user. Follow all local, state, and federal biohazard-handling regulations for disposal of the contents of the waste reservoir.

Prevent waste-reservoir overflow by emptying the container when the waste indicator indicates that it is full.

Run the instruments sterilize routine after each day's use. Note that this procedure has not been proven to result in microbial sterility.

Sécurité Biologique

Biorisques: L'FlowSight est évalué à un niveau de sécurité biologique L1. Ne pas acquérir ou vider des échantillons contenant des agents infectieux sans les avoir inactivés. Il est recommandé que les échantillons soient fixés dans du paraformaldéhyde 2% pendant au moins 10 minutes avant d'acquérir des échantillons avec l'FlowSight.

L'utilisation, le confinement et l'élimination des matériels biologiques dangereux sont tenus d' tre en conformité avec les normes de sécurité relatives au laboratoire et de la directive 93/95/E et restent sous la responsabilité de l'utilisateur. Respectez la réglementation en vigueur pour le traitement et l'élimination des déchets dans des réservoirs prévus à cet effet.

Prévenir l'accumulation des déchets en vidant le réservoir lorsque l'indicateur indique qu'il est plein. Stériliser les instruments de routine apr s chaque journée d'utilisation. Notez que cette procédure ne garantit pas la stérilité vis à vis des microbes. Le Niveau de sécurité biologique pour l'instrument est de niveau L1.

Spare Parts

The instrument contains no serviceable parts. Only Amnis-trained technicians are allowed to repair, maintain, and set up the alignment of the laser beams.

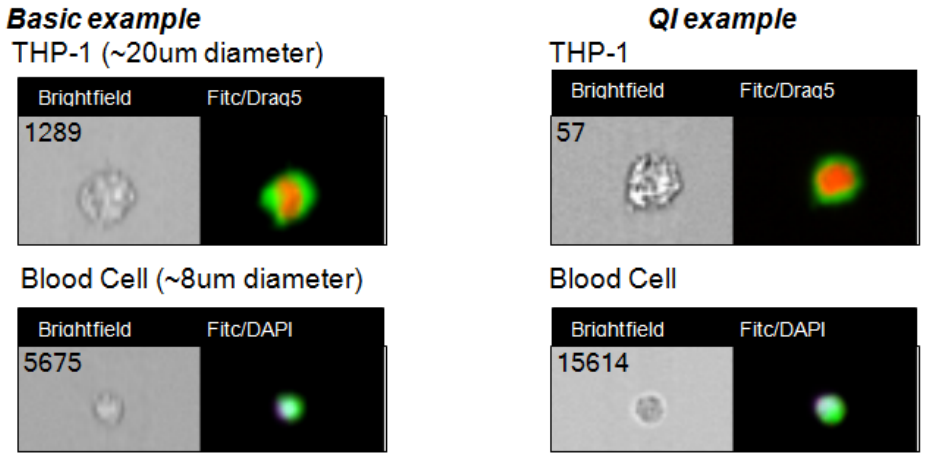
Chapter 2: Experimental Design

Sample Preparation

Experimental Design

The FlowSight system can quantify the intensity, specific location, and distribution of signals within tens of thousands of cells per sample. The system can perform most standard flow cytometric assays, and can also leverage the technology's imaging capabilities to discriminate image-based changes within individual cells and cell populations.

1. **Choice of Cell Type:** The cell/particle size should be less than 60 microns in diameter.



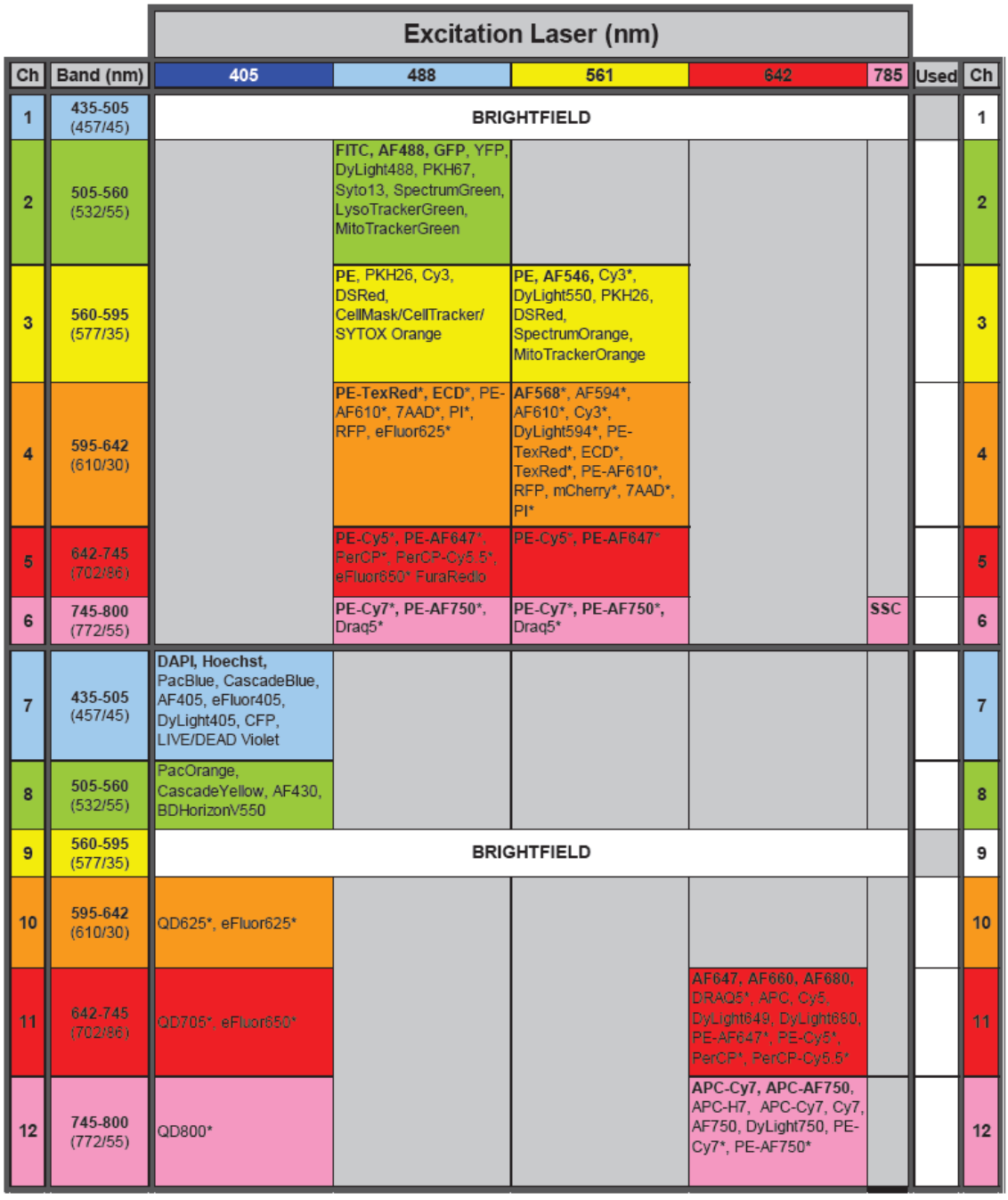
2. **Final Sample Concentration and Volume:**The recommendation is at least 1 million cells in 50 µl ($2x10^7$ cells/ml) in PBS/2%FBS in a 1.5ml siliconized microcentrifuge tube.
3. **Protocols:** In general, any established labeling protocol used for flow cytometry will work with the FlowSight (see Current Protocols in Cytometry for general labeling techniques). Stain cells on ice in the presence of azide when possible to reduce non-specific capping of antibody. Use polypropylene tubes, preferably siliconized, to process samples.
4. **Choice of Fluorochromes:** Choose fluorochromes that are excited by the lasers in your FlowSight (405, 488 and 642 nm are most common). Use the chart below or look online for a spectra viewer that will help you plan which dyes will work best. Channel 1/9 are the most common channels for brightfield. SSC imagery may be placed into channel 6 if desired. Dyes with an * are excited by at least one laser directed to channels 1-6 and another directed to channels 7-12. For these dyes, the channel that the dye will

appear brightest in depends on the relative laser powers used. Recommended dyes are indicated in boldface.

5. **Compensation:**Have a sample of cells each labeled with a single-color for each fluorochrome used (i.e. FITC only cells, PE only cells, etc.)
6. **Cell Aggregation:**Minimize aggregation problems by straining the sample through a 70 micron nylon mesh strainer or by using an anti-clumping buffer such as EDTA or Accumax prior to fixation.
7. **Fixation:** If fixation is desired, thoroughly fix cells with 1% formalin on ice for 20 minutes.
8. **Number of samples:** No more than 30 total for feasibility experiments. Please limit the samples to the following; Positive and Negative **biologic controls**, **compensation controls**, and experiment samples.
9. **Brightness of Stain and Stain Balancing:** Quantifying the location and distribution of signals in an image is a demanding task that requires optimized labeling. Here are a few suggestions to help design the experiment:

    - Try to achieve at least a full log shift in fluorescence, as measured by a standard flow cytometer.
    - Use the brightest dye for the antigen with the smallest copy number
    - The brightness of probes can be independently controlled by changing the laser power. However data quality is enhanced when the brightness levels of all probes excited off a single laser are balanced to within a log of each other. Probe balancing avoids the saturation of bright stains when they are combined with dim stains in the same sample.

FlowSight Fluorochrome Charts



Recommended dyes (based on optimal excitation and detection channels) are in boldface. Ideal dyes with 1, 2 or 3 laser systems:

1 laser (488): AF488, PE, PE-TxRed, PE-Cy5, PE Cy7, SSC-Ch6.
2 laser (488, 642): AF488, PE, PE-TxRed, SSC-Ch6, AF647, APC-Cy7
3 laser (488, 405, 642) AF488, PE, PE-TxRed, SSC-Ch6, DAPI, AF647
Band-pass filter values assume 3 laser configuration.

Chapter 3: Using the FlowSight

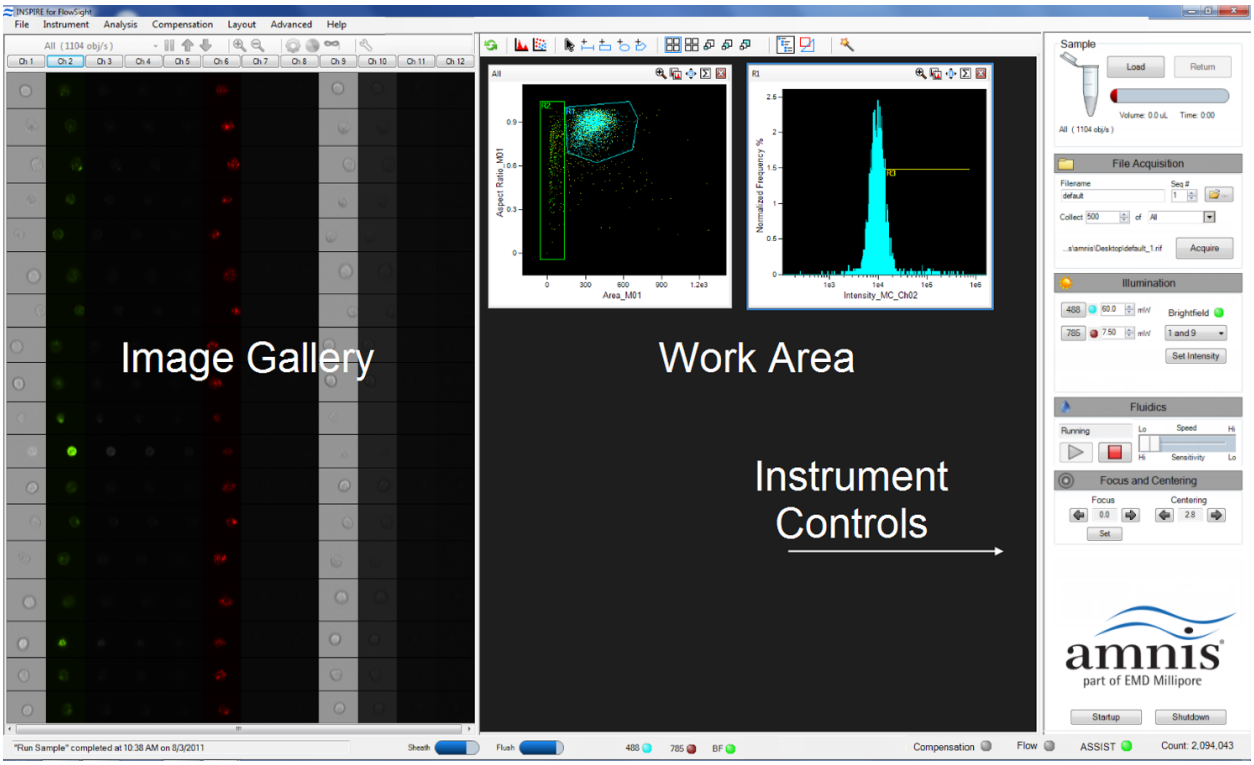
Operating the FlowSight

This chapter describes the operation of the FlowSight system using the INSPIRE for FlowSight software. Daily operation involves an initialization of the system and calibration using the Calibration bead reagent, followed by sample runs and data acquisition, and finally a shutdown procedure which sterilizes the system and prepares for the instrument for use the following day. Optimizing instrument setup for sample runs is also described in this chapter.

- User Interface
- Reagents
- Daily Calibration and Testing
- Instrument Setup
- Data Acquisition
- Daily Shutdown
- Autosampler
- Quantitative Imaging

FlowSight User Interface

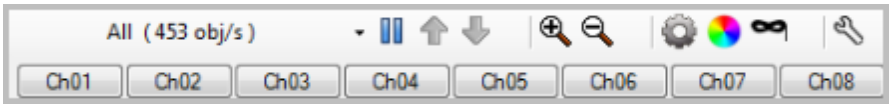
The user interface is divided into 3 areas, the image gallery where channel images are displayed, a work area where graphs of features are displayed and the controls section where the instrument is controlled. Status information is displayed along the bottom of the window.



The Image Gallery

Images are displayed in the image gallery during setup and acquisition.

Image Gallery Tools



Icon	Name	Description
All (911 obj/s)	All	Select the population to view
	Pause	Pause/Resume the display
	Up/Down	Move up or down in the image gallery while paused
	Zoom in	Enlarges the imagery

	Zoom out	Resets the zoom
	Settings	Adjust the image display and channels collected
	Color	Turn color ON/OFF
	Mask	Displays the segmentation mask on the images
	wrench	Tools to measure pixel intensity of displayed images
Ch02 Ch03	channel name	Clicking on a channel name will also open the set-tings tool

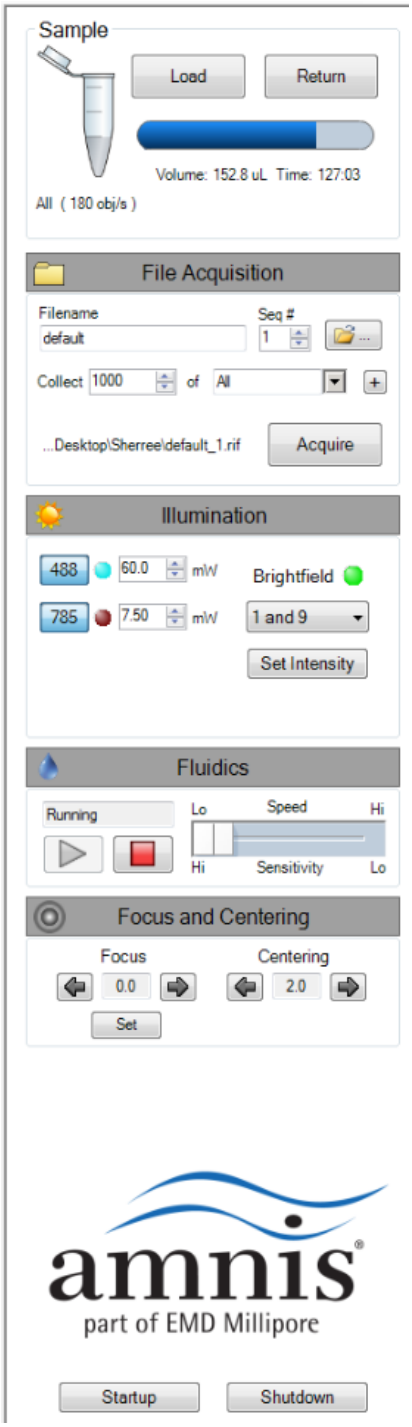
The Analysis Area

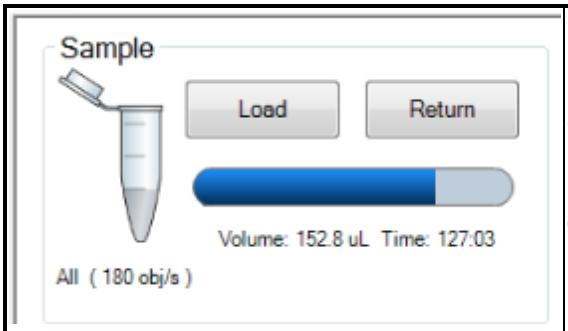
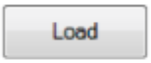
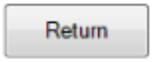
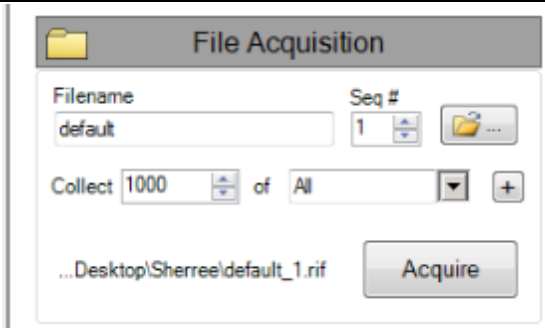
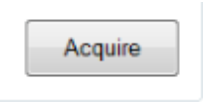
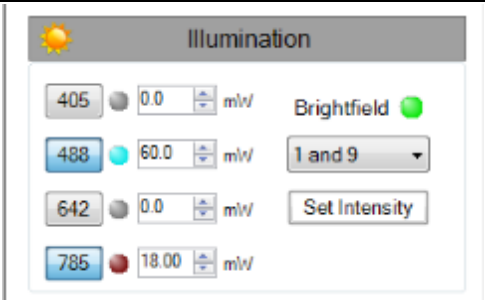
Graphs are displayed in the analysis area during setup or acquisition. Regions can be drawn on the graphs to create populations.

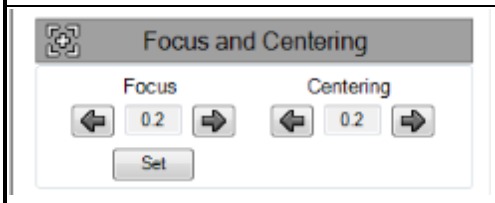
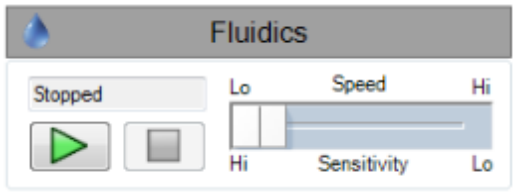
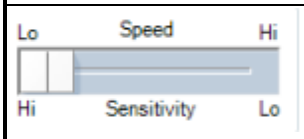
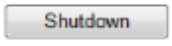
Icon	Name	Description
	Reset	Refreshes the graphs with incoming data
	Histogram	Create a histogram tool
	Scatter Plot	Create a bivariate scatter plot tool
	Pointer	Reset cursor to pointer
	Line region	Draw a line region on a histogram
	Rectangle region	Draw a rectangular region on a scatterplot
	Oval region	Draw an oval region on a scatterplot
	Polygon region	Draw a poygon region on a scatterplot
	Select All	Selects all plots in analysis area
	Tile	Tiles the graphs in the analysis area to fill the space
	Size Plots	Sets size of selected plots to small, medium or large
	Populations	Opens the population manager
	Regions	Opens the region manager
	Wizards	Opens the list of wizards (compensation)

The Instrument Control Panel

The instrument control panel provides tools to control instrument operation, data acquisition and status.



	In the Sample section you can load a sample or return a sample. Sample volume and time remaining is displayed and the rate of the selected population when a sample is running.
	Loads the sample
	Returns the sample
	In the Acquisition Settings section you can type in a custom filename, set the sequence #, choose the data file folder, type the number of events and choose the population to collect.
	Begin Acquisition
 (After Acquisition begins)	Pause acquisition
 (After Acquisition begins)	Stop acquisition
Custom Filename Text	Type the filename
Seq#	Choose the beginning sequence number
	Navigate to the folder to save the data
Collect	Enter the number of events to collect
of	Choose the population to collect
	Add a second population to collect
	In the Illumination section you can turn laser and brightfield illumination on or off and set intensities. All lasers have variable power and are defined by their excitation bandwidth.

	405nm laser excitation - currently set to OFF and 0 mW of power.
	488nm laser excitation- currently set to ON at 60 mW of power.
	642nm laser excitation- currently set to ON at 150 mW of power.
	785nm laser excitation- currently set to OFF at 5.72 mW of power. This laser is for side scatter only.
	Brightfield illumination is shown as ON in channels 1 and 9.
	Sets the Intensity of the brightfield to 800 counts.
	Focus and Centering can be adjusted using the right and left arrows.
	Runs the focus pan test and sets focus automatically.
	Adjust the speed and sensitivity for the run.
	Run fluidics.
	Stop fluidics.
	Speed and Sensitivity are inversely related.
	Runs the startup script.and initializes fluidics
	Runs the shutdown script and sterilizes the system.

Reagents

Sterilizer, Cleanser, and Debubbler

These recommended reagents have been formulated to optimize the performance of the FlowSight seals, valves, syringes, and lines. The use of the recommended reagents is required for proper operation of the instrument. The Sterilizer, Cleanser, and Debubbler reagents are used in the Sterilize and Debubble scripts.

Reagent	Name	Source*	Catalog#
Sterilizer	0.4-0.7% Hypochlorite	VWR	JT9416-1
Cleanser	Coulter Clenz®	Beckman Coulter	8546929
Debubbler	70% Isopropanol	Millipore	1.37040
Sheath	PBS, $Ca^{++}Mg^{++}$ free	Millipore	BSS-1006-B (1X) 6506-1L (10X)
Rinse	Deionized water		
Calibration Beads	FlowSight Calibration Beads	Amnis	400300

*provided for information only, other sources of the same reagent may be used.

Waste Fluid

The two liter waste bottle holds all of the fluids that have been run through the FlowSight,. Add 200 ml of bleach to the empty waste tank. It is recommended that the waste bottle contain 10% bleach when full.

Sheath Fluid

Two bottles are provided: one labeled Sheath to be filled with phosphate buffered saline (PBS with no surfactants) for running samples and one labeled Rinse to be filled with de-ionized (DI) water for rinsing the instrument during shutdown. Fluid is drawn from these bottles into the sheath and flush syringe pumps. The sheath pump helps to control the speed of the core stream and the size of the core stream diameter. The flush pump is used to clean and flush the system.

Fluid level warnings

Sensors behind the bottles trigger a pop-up window to alert the user that the volumes are low or the waste volume is high. The triggers are set such that any script currently running can finish. Do not remove a bottle during the script. Once the script has finished, remove the bottle and fill the appropriate solution. Waste can be emptied at any time. Dispose of the waste in accordance with local regulations.

The left window below is indicating that the Sterilizer solution is low and needs to be filled. The right window is indicating all bottles are currently OK.

Daily Startup and Calibration

This section describes how to prepare the FlowSight for use. The FlowSight can be left on with INSPIRE launched, but the following instructions also describe how to turn on the FlowSight if the power is off.

Turning on the FlowSight

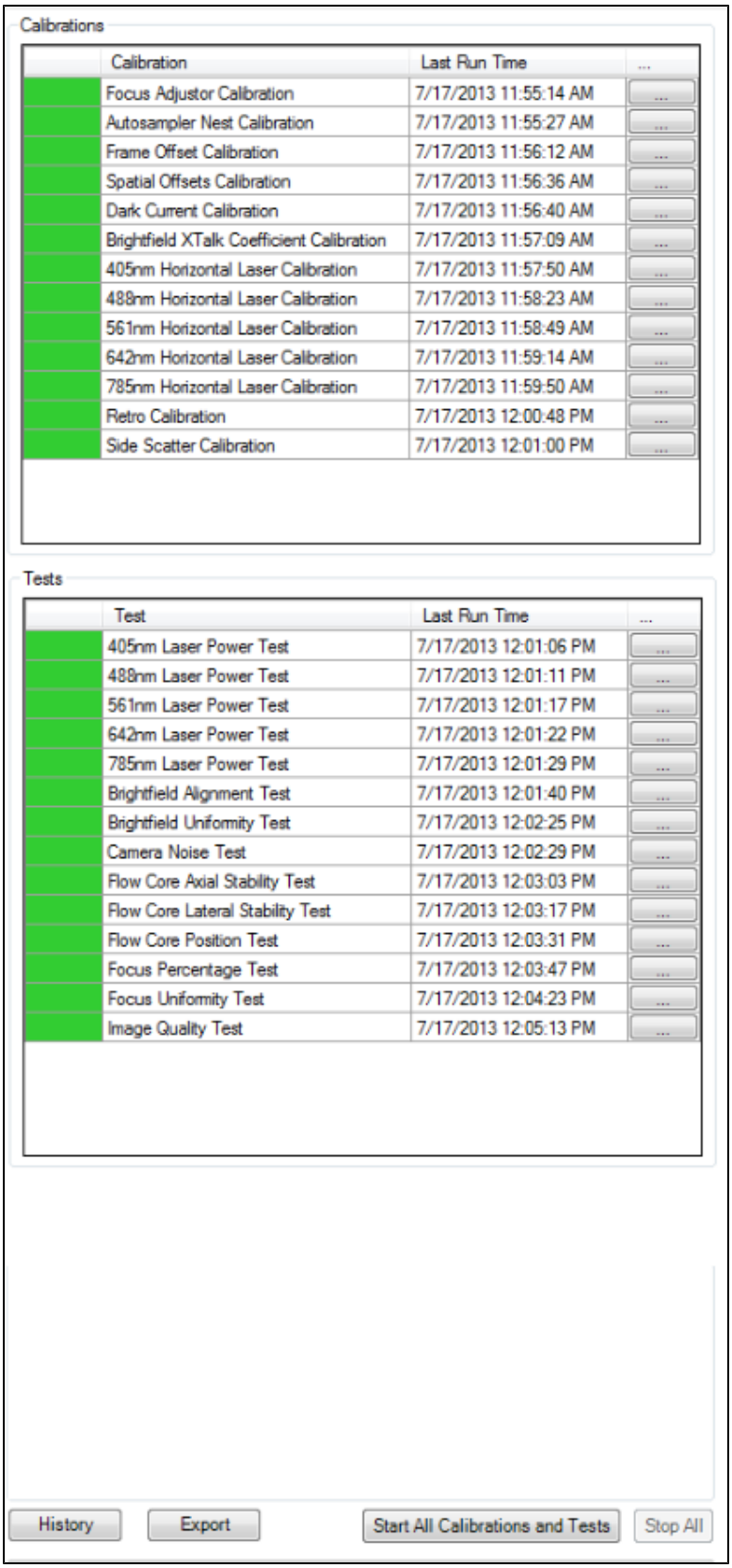
1. Turn on both computers. The large Linux- based computer runs the imaging and the small Windows computer runs the user interface. Toggle the power switch on the right lower panel of the instrument from OFF to ON.
2. Log on with the user name (Amnis) and password (IS100).
3. Launch the FlowSight software using the shortcut on the desktop after the computers and instrument have started.

Preparing to run calibration

4. Fill the rinse bottle with deionized water and the sheath bottle with PBS.
5. Empty the waste tank. Push on the quick-disconnect buttons to remove the tubing from the waste tank. Add 200 ml of bleach to the two liter waste bottle. The final bleach concentration for a full waste tank should be 10%for sterilization.
6. Click **Startup**. This script fills the system with sheath and flushes out all of the storage rinse that was in the system. (~12 minutes)
7. Click **Load** and place a tube containing 2 drops of FlowSight Calibration reagent on the uptake port. Vortex the reagent before use.
8. Click **Start All Calibrations and Tests** at the bottom of the ASSIST view. The calibrations and tests will run and the results saved to the database.

   Note: Instrument calibrations may also be run individually by selecting a particular procedure under Calibrations or Tests. Next to each is a green or red rectangle. If the procedure fails, it turns red. If a procedure fails, repeat it. If it fails twice, see the troubleshooting chapter 4 or call your Amnis Field Service Representative.

9. When the calibrations and tests have all passed, close the ASSIST window. This window can be re-opened under the Instrument menu.


Calibrations
Calibration
Last Run Time
...
Focus Adjustor Calibration
7/17/2013 11:55:14 AM
Autosampler Nest Calibration
7/17/2013 11:55:27 AM
Frame Offset Calibration
7/17/2013 11:56:12 AM
Spatial Offsets Calibration
7/17/2013 11:56:36 AM
Dark Current Calibration
7/17/2013 11:56:40 AM
Brightfield XTalk Coefficient Calibration
7/17/2013 11:57:09 AM
405nm Horizontal Laser Calibration
7/17/2013 11:57:50 AM
488nm Horizontal Laser Calibration
7/17/2013 11:58:23 AM
561nm Horizontal Laser Calibration
7/17/2013 11:58:49 AM
642nm Horizontal Laser Calibration
7/17/2013 11:59:14 AM
785nm Horizontal Laser Calibration
7/17/2013 11:59:50 AM
Retro Calibration
7/17/2013 12:00:48 PM
Side Scatter Calibration
7/17/2013 12:01:00 PM
Tests
Test
Last Run Time
...
405nm Laser Power Test
7/17/2013 12:01:06 PM
488nm Laser Power Test
7/17/2013 12:01:11 PM
561nm Laser Power Test
7/17/2013 12:01:17 PM
642nm Laser Power Test
7/17/2013 12:01:22 PM
785nm Laser Power Test
7/17/2013 12:01:29 PM
Brightfield Alignment Test
7/17/2013 12:01:40 PM
Brightfield Uniformity Test
7/17/2013 12:02:25 PM
Camera Noise Test
7/17/2013 12:02:29 PM
Flow Core Axial Stability Test
7/17/2013 12:03:03 PM
Flow Core Lateral Stability Test
7/17/2013 12:03:17 PM
Flow Core Position Test
7/17/2013 12:03:31 PM
Focus Percentage Test
7/17/2013 12:03:47 PM
Focus Uniformity Test
7/17/2013 12:04:23 PM
Image Quality Test
7/17/2013 12:05:13 PM
History
Export
Start All Calibrations and Tests
Stop All

Data Acquisition

After the FlowSight system is calibrated, you are ready to acquire experiment data files. The sample is loaded into the sample pump. Sample is injected into the flow cell to form a single core stream that is hydrodynamically focused in front of the imaging objective. Refer to the Autosampler upgrade section below for using unattended operation.

Refer to the Sample Preparation Guide or Chapter 2 for experimental set-up recommendations. Use compatible sample solutions from the table below.

Sample Solution	Sheath Fluid	Acceptable
PBS	PBS	Yes
PBS	Water	Yes*
PBS/Surfactant	PBS	Yes
PBS/Surfactant	Water	No
Water	PBS	Yes
Water	Water	Yes
Water/Surfactant	PBS	No
Water/Surfactant	Water	Yes

* Cells in PBS run with water sheath will swell.

Sample order:

Samples from an experiment are typically run in the following order:

- Experimental sample with the brightest stains to set the sensitivity for the run
- Single color DNA dye control NO BF or SSC to ensure correct dye concentration
- 10% bleach to wash out DNA dye followed by PBS
- Single color fluorescence compensation samples (no DNA dye) NO BF or SSC, all channels enabled
- The rest of the experimental samples with DNA dye (if applicable)

Loading and running the sample:

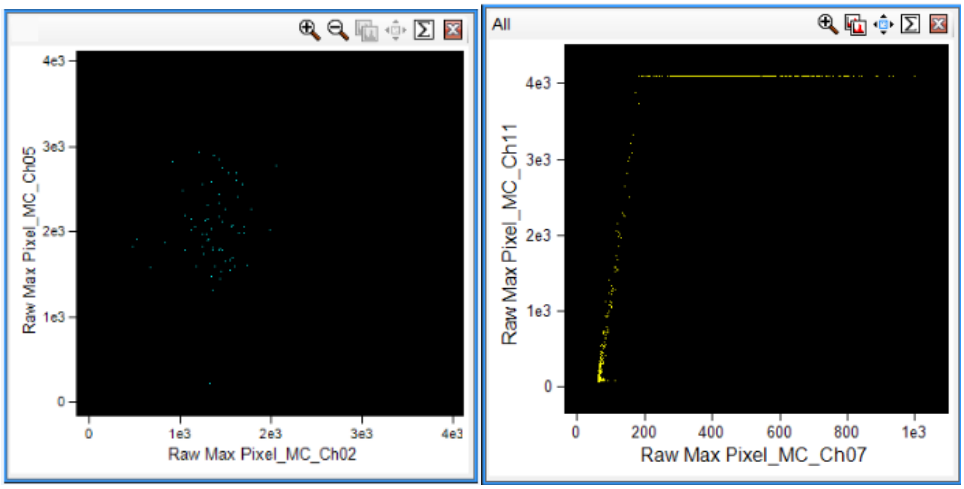
1 Press **Load**, and load an aliquot of the brightest sample in the experiment, that fluoresces with each fluorochrome used. It is critical that you run this sample first to establish the instrument settings. (DO NOT change laser settings for the experiment once established on this sample.)

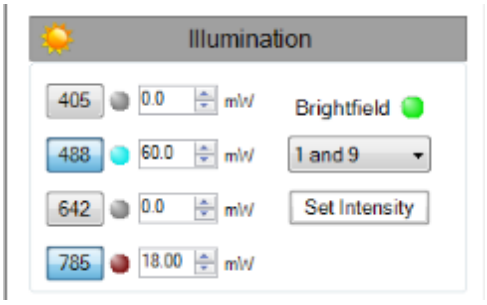
Note: Application-specific instrument settings can be saved in a template and used to facilitate instrument setup, but it is recommended that you verify the appropriateness of the settings for the specific experimental run.

2 Turn on each laser used in the experiment by clicking on the wavelength. Create scatter plots of Raw Max Pixel for the channels used in the experiment. Set the laser powers so each fluorochrome has Raw Max Pixel Intensities between 100

and 4000 counts, as measured in graphs and there is no saturation. The default saturation color can be changed by clicking Channels in the Acquisition section.

In the examples below the objects are not saturating in channels 2, 5 or 7 but saturating in channel 11 indicated by the events lining up at 4095 counts.





3 Select Brightfield channels. Default is Channels 1 and 9. Click **Set Intensity**.

4 Set the display properties if needed. See Setting the Image Display Properties

5 Create graphs to gate on cells of interest.

Recommended: Scatterplot of *Area* versus *Aspect Ratio* of brightfield to gate on cells and eliminate debris. Scatterplots or histograms of *Raw Max Pixel* for the channels used in the experiment for setting laser power. Scatterplots or histograms of Intensity for the channels used in the experiment.

To identify objects for inclusion in or exclusion from the acquiring data file the following features in any channel are available:

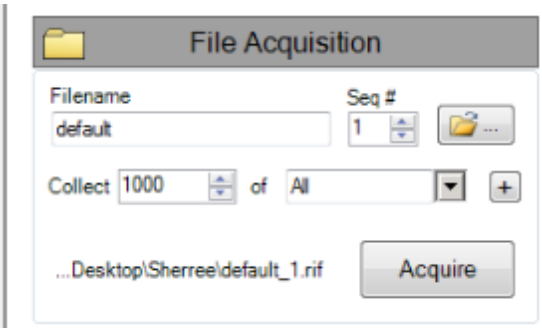
- **Area:** The number of pixels in an image reported in square microns.
- **Max Gradient Intensity:** The value of the largest slope spanning three pixels in an image. This feature measures image contrast or focus quality.
- **Intensity:** The integrated intensity of the entire object image; the sum of all pixel intensities in an image, background subtracted.
- **Mean Pixel:**The average pixel intensity in an image, background subtracted.
- **Raw Max Pixel:**The intensity value of the brightest pixel in an image.
- **Raw Min Pixel:** The intensity value of the dimmest pixel in an image.

- **Saturation Count:** The number of pixels in an image that have an intensity value of 4096.
- **Saturation Percent:**The percentage of pixels in an image that have an intensity value of 4096.

Collecting and saving the data files

Once the sample is running and the FlowSight is properly set up, you are ready to acquire the data as a raw image file (.rif). This file contains uncompensated pixel data along with instrument settings and ASSIST information in a modified TIFF format. The file includes only those objects defined by the population selected in the acquisition section. As an option, a compensation matrix may be applied to the data as it is collected by either going through the compensation wizard to create the matrix or choosing a matrix from the File menu.

The setup can be saved as a template file (.ist) for future experiments.



6 Enter the number of cells you want to acquire after **Collect** and select the population. To add another population click the + box.

7 Select the channels to collect if desired. (See Display Properties below)

8 Enter the file name.

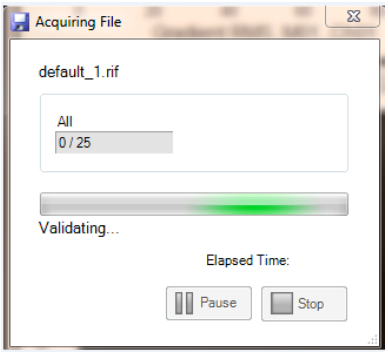
The number in the **Sequence #** box is appended to the file name, followed by the **.rif** extension. The sequence number increases by 1 with each successive data acquisition. Files collected with BF off will be appended with noBF. File names must be 256 or fewer characters in length, including the path and file extension. In addition, file names cannot contain the following characters: \,/,:,*,<,>, or |.

9 Browse to select an existing folder or to create a new folder in which to save the files. Note: do not store data on the imaging computer.

10 Click the Acquire button to acquire the data if desired to collect this file.

11 The data file(s) are automatically saved in the selected folder once the desired number of objects are collected.

To prematurely stop acquisition click  . The system prompts you to either discard the acquired data or to save the collected data in a file. The acquisition can be paused and resumed by clicking  .

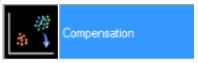


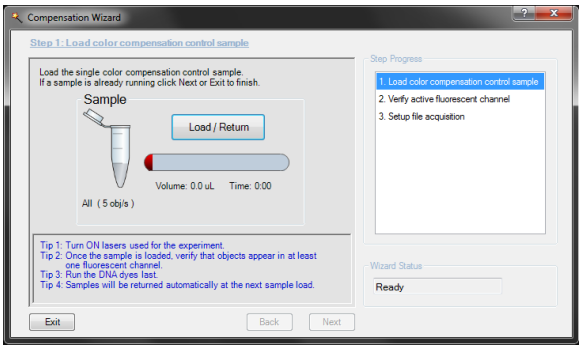
12 Once acquisition finishes, either press Load to load the next sample or Return return the remaining sample.

Note: If the next sample has no nuclear dye and follows a DNA intercalating dye-stained sample, run **Load** a solution of 10% bleach and then PBS to ensure that residual dye does not stain the subsequent samples.

Collecting compensation files

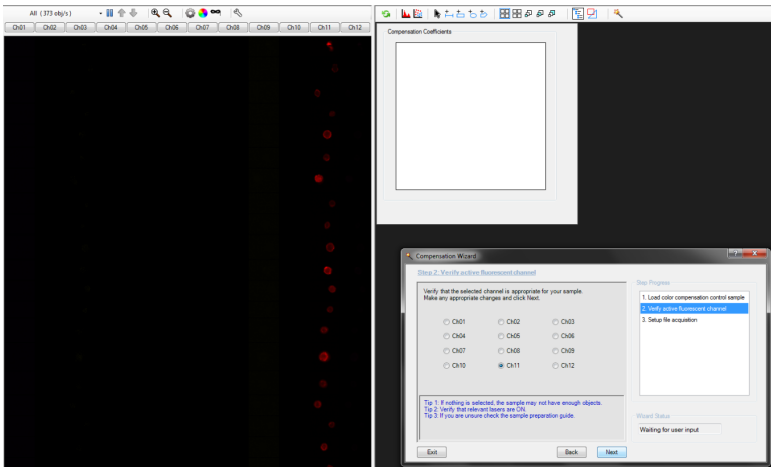
The compensation samples are collected using the compensation wizard or may be collected manually by turning brightfield and the 785 scatter laser OFF and enabling all channels. In either case, the experimental data files may be compensated in IDEAS during analysis. Collect compensation files with the same laser power settings as the experimental samples.

13 To use the compensation wizard click  to open the wizard list and select Compensation. 

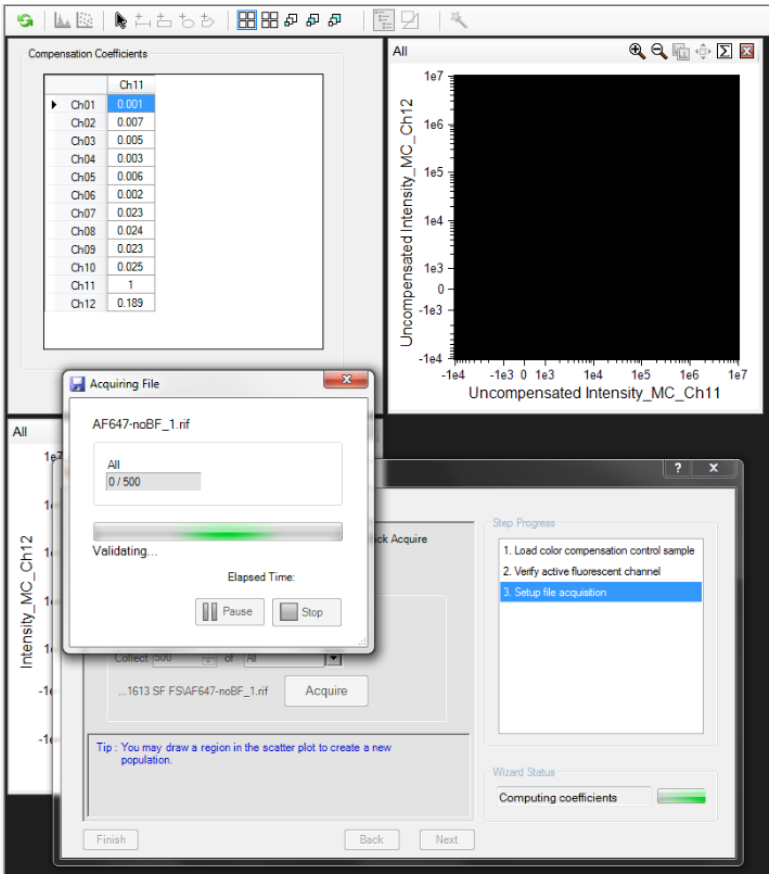


14 Click **Load/Return** or **Compensation Sample Load** (depending on your interface) to run the first compensation single-color sample. Note: if sample is already running prior to launching the wizard, go directly to the next step.

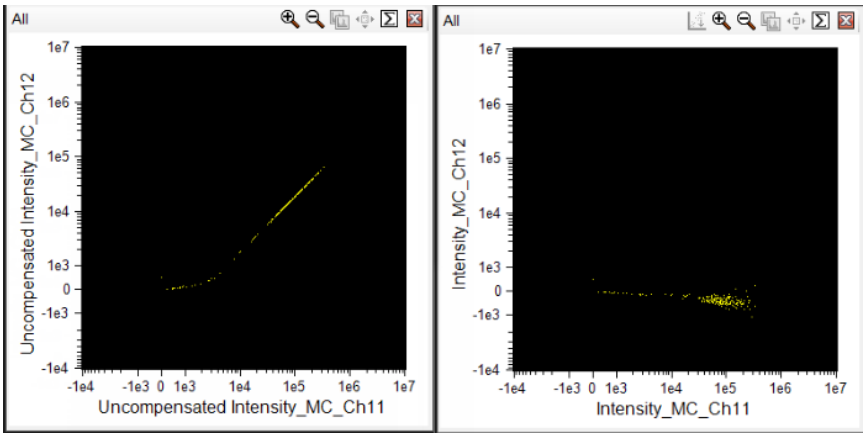
15 Click **Next** when the sample is running. The wizard will turn the BF and 785 nm illumination OFF and enable all 12 channels.



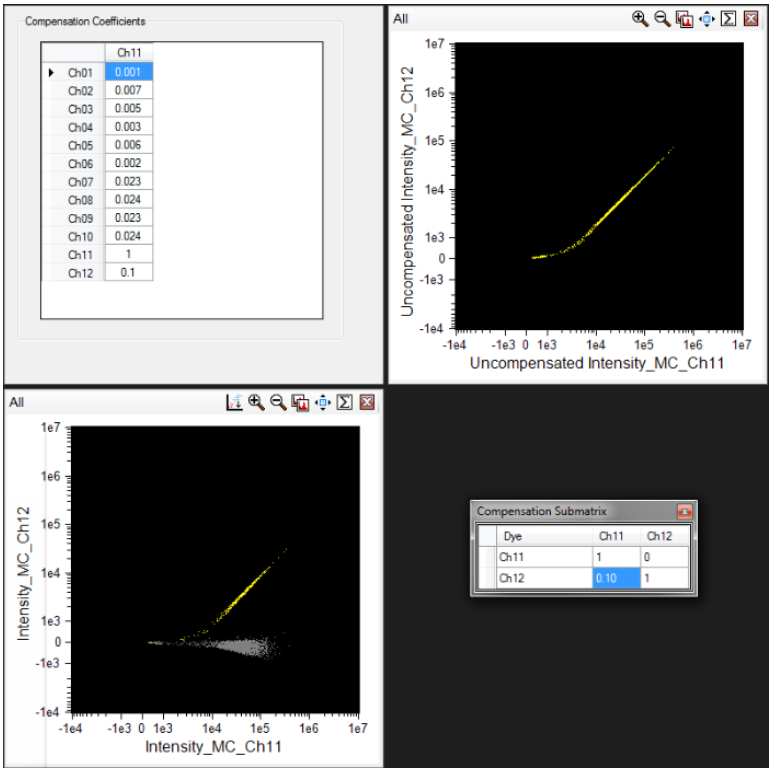
16 Verify the channel for the sample loaded and click **Next**. The compensation coefficients are calculated and added as shown in the table. Compensation is applied to the Intensity feature and images. Two scatter plots are added to the work area. One of Uncompensated Intensity and one of Intensity for the peak channel vs. the adjacent channel is added to the work area.



17 If desired, compensation values can be adjusted by clicking on the compensation tool button in the graph. 

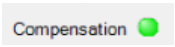


For example changing the matrix value in the 2x2 submatrix from 0.189 to 0.10 changes the compensation as shown from the graph above (properly compensated) to the graph below (under compensated).



18 Repeat from step 13 for each single-color compensation sample in the experiment.

19 Click Finish and save the matrix (.ctm) when done.

The previous settings will be restored and the compensation matrix is now being applied to the Intensity features and images. This is indicated by the compensation button in the lower task bar 

Note: Uncompensated pixel values will still be collected. You are now ready to continue collecting the rest of the experimental samples.

Note: The experimental template is reloaded when the compensation wizard is finished. If you are collecting compensation files without using the wizard be sure

to turn the 785 scatter laser back ON, brightifield ON, and select the channels to acquire if desired.

20 Press **Load** and load the next experimental sample.

21 Enter the number of cells you want to acquire after **Collect** and select the population.

22 Change the file name for the sample.

23 The number in the **Sequence #** box is appended to the file name, followed by the **.rif** extension. The sequence number increases by 1 with each successive data acquisition.

24 Click **Acquire** to collect the file when the cells are running smoothly.

25 Repeat for each sample.

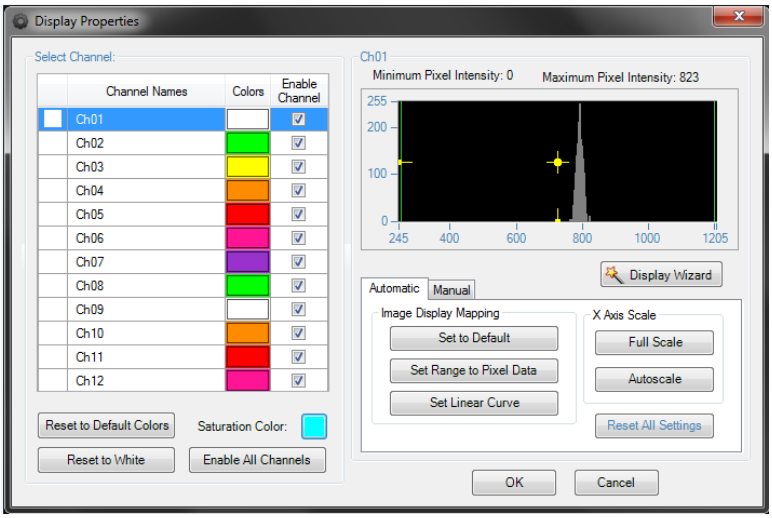
26 When finished running samplesfor the day press **Shutdown**,

Setting the Image Display Properties

1 Click on the settings tool  or a channel name to open the window.

2 Here you may:

- Select the color for image display of each channel.
- Select the color for displaying saturated pixels.
- Set the display intensity mapping.
- Enable channels to collect.
- Run the display wizard which helps to map the display intensities.



Note: Mapping the **Display Intensity** settings on the graph, value of the display (the Y Axis) to the range of pixel intensities (the X axis) is done using the green limit lines. The range of pixel intensities will from the camera is 0-4095. The limits of the graph enable you to use the full dynamic range of the display to map the pixel intensities of the image. **Note:** Changing the display mapping does not change the data. They are for display purposes only.

Setting up the work area

Create scatterplots or histograms with the tools in the analysis area.

The quantitative features that are available to graph the data are listed in the table below. Features are algorithms that are applied to a specific region of interest of the image defined by a segmentation mask and image pixel values defined by the channel image. Segmentation masks are named M01 through M012 indicating intensities above background in the respective channel. MC is the combination of all of the individual channel masks. Feature names are described by the Feature_ Mask_Image. For example Intensity_M02_Ch02 indicates the sum of all channel 2 pixel intensities in Mask M02.

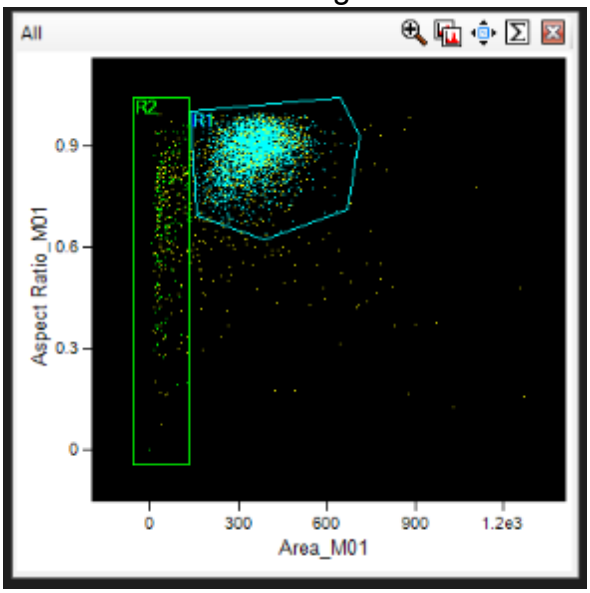
Note: See the IDEAS User Manual for more information on features and graphing. The tools functionality is the same.

Features available for FlowSight

Name	Mask_Channel	Definition
Area	M01-M012,MC	The size of the mask in square microns.
Aspect Ratio	M01-M012	The ratio of the Minor Axis divided by the Major Axis.
Bkgd Mean	Ch01-Ch12	The average intensity of the camera background.
Bkgd StdDev	Ch01-Ch12	The standard deviation of the background intensities.
Camera Line Number	none	An incremental count of objects.
Camera Timer	none	The clock rate in KHz. This is relative time.
Gradient RMS	M01_Ch01 through M12_Ch12	Enumerates changes of pixel values in the image to measure the focus quality of an image.
Intensity	MC_Ch01 through MC_Ch12	The sum of the pixel intensities in the mask, background subtracted.
Major Axis	M01-M012	Describes the widest part of the mask.
Mean Pixel	M01_Ch01 through M12_Ch12	The average pixel value within the mask, background subtracted.
Minor Axis	M01-M012	Describes the narrowest part of the mask.
Object Number	none	The sequence of objects.

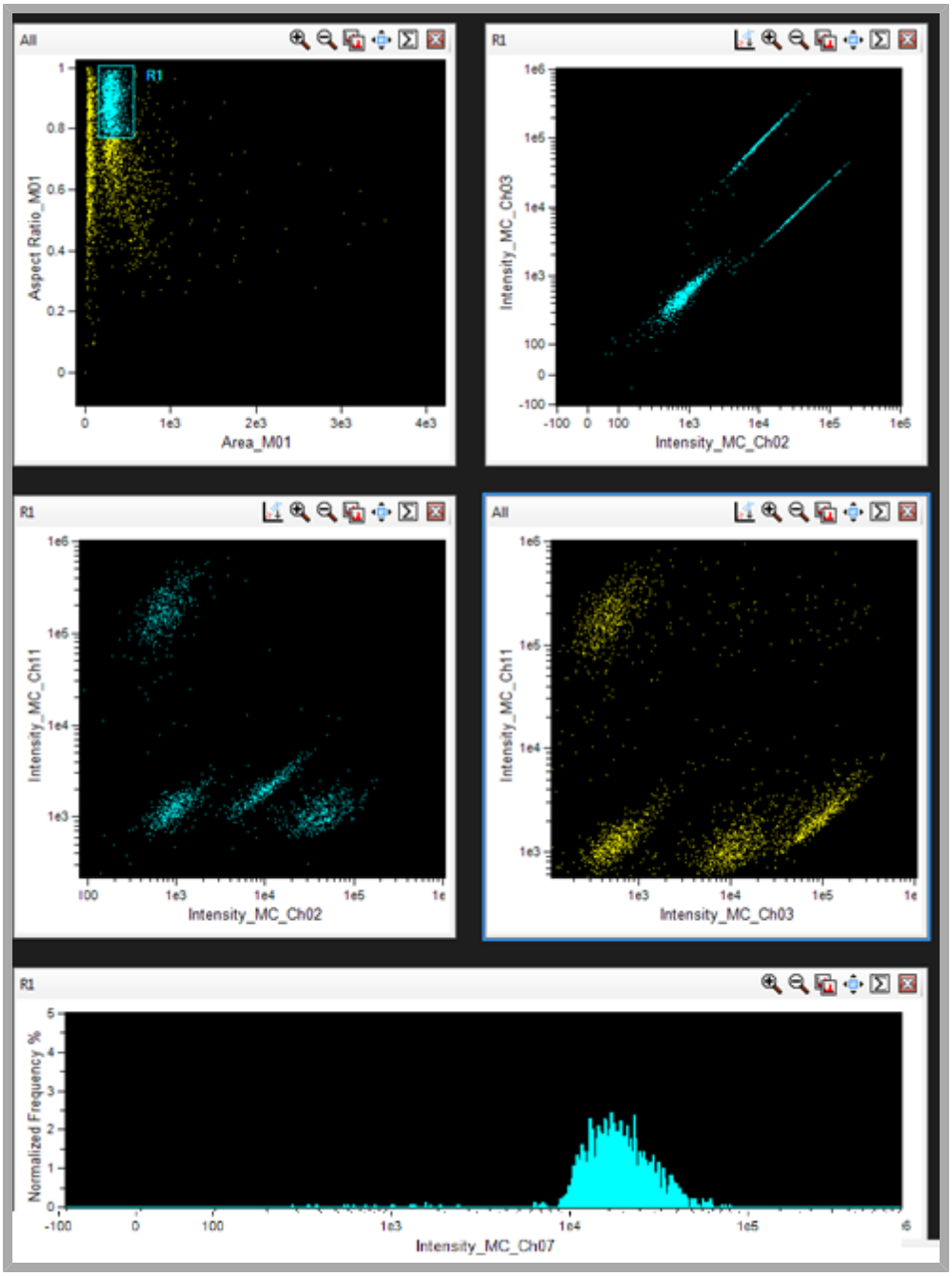
Name	Mask_Channel	Definition
Raw Centroid X	none	The central tendency of the pixels along the X Axis and Y Axis, respectively.
Raw Centroid Y	none	The central tendency of the pixels along the X Axis and Y Axis, respectively.
Raw Max Pixel	MC_Ch01 through MC_Ch12	The largest pixel value.
Raw Min Pixel	MC_Ch01 through MC_Ch12	The lowest pixel value.
Uncompensated_ Intensity	MC_Ch01 through MC_Ch12	The sum of the pixel intensities in the mask, background subtracted, no compensation applied.

1. For a standard experiment create a scatterplot of Area versus Aspect Ratio of the brightfield channel and draw a region around the single cells.
   A. Click the scatter plot tool and choose the population All, the X axis feature Area_M01 and Y axis Feature Aspect Ratio_M01 (for brightfield in channel 1).
   B. Click the polygon region tool and click on the graph to create the region. A population is created and the population becomes available in the list at the top of the image gallery.
   C. Single cells will have an aspect ratio around 1. The Area of the debris is small and doublets or aggregates will be larger. In this example R1 is drawn around the single cells and R2 is debris.



2. Confirm your gating by viewing the populations created by gates in the image gallery.
3. Create scatterplots and histograms as required.

Example template for 4 color experiment



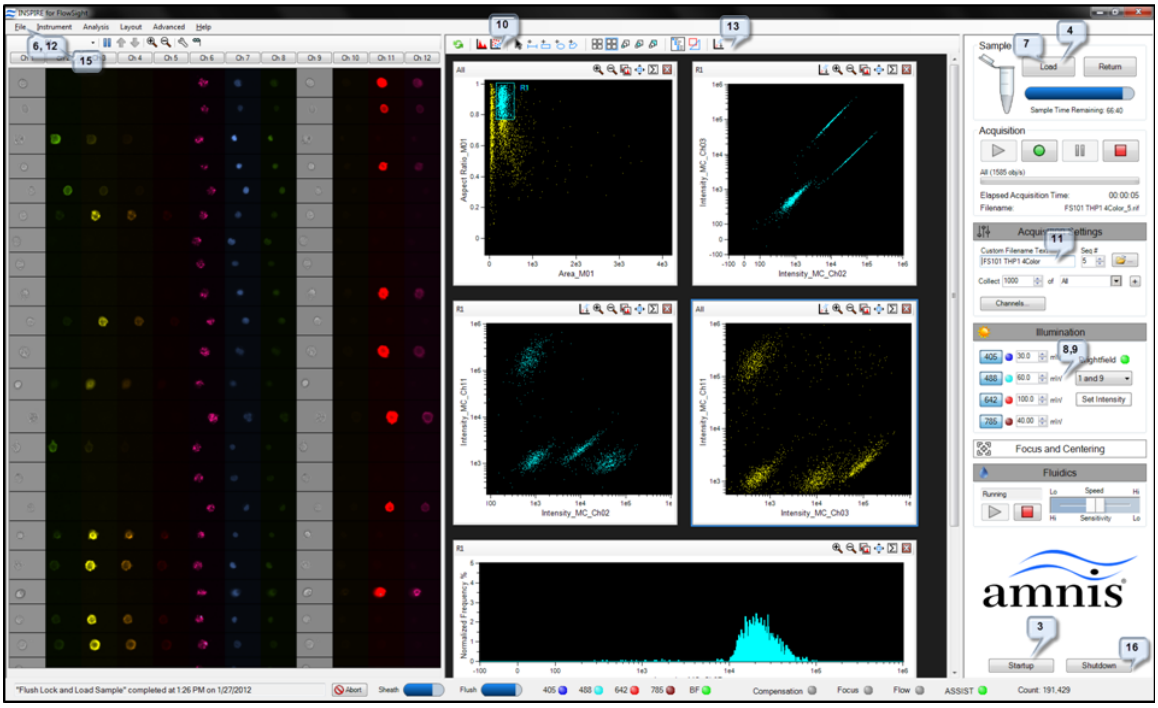
Refer to the IDEAS User Manual for more detailed information on graphing tools, moving regions or panels or managing populations.

Quick Start Guide to FlowSight Operation

1 Power up the system and launch the FlowSight application.
2 Check to be sure the buffer containers are full and the waste tank is empty.
3 Select Startup and the instrument will flush the system and load sheath in ~12min.
4 Press Load and load two drops of FlowSight calibration beads. Vortex first.
5 In the ASSIST view, press Start all calibrations and tests. Calibration takes ~12min and when all tests pass the system is ready to run for the day.
6 Select load default template or experiment template from the File menu.
7 Press Load and load an aliquot of a sample with each fluorochrome present. Run on 'Low' speed for best sensitivity.
8 In the Illumination section, turn on the appropriate lasers for each fluorochrome in the experiment.
9 Adjust the laser power to maximize brightness and prevent saturation. The 405 laser power should be adjusted to use a minimal acceptable power to prevent autofluorescence and PE excitation into the BF9 reference channel.
10 Create dot plots and regions to identify the cells to collect, or collect 'All' events.
11 Set the acquisition parameters including file name, destination folder, number of events and population(s) to collect.
12 Choose file format, either .rif (IDEAS), .fcs (FACS software) or both from File menu.
13 Compensate data if needed. Data can be recompensed in IDEAS post acquisition.

A. Open the compensation wizard.
B. Load a compensation single color sample.
C. Verify the system finds the correct channel.
D. Set the population to save.
E. Set the acquisition file name and destination.
F. Press next to save the data file.
G. Repeat steps B through F for each compensation sample. Press exit and save to return to the experiment.

14 Continue collecting all experiment files using consistent instrument settings *(In general brightfield will be in channels 1 and 9, SSC ~40mw in channel 6; and the cells to collect R1 using brightfiled area vs. aspect ratio to identify single cells).*
15 Save an experiment template by selecting Save Template from the File menu.
16 Shut the system off by pressing the Shutdown button. The system sterilizes in ~40 min.


6, 12
15
10
13
7
4
11
8,9
3
16
Sample
Load
Return
Acquisition
Illumination
Focus and Centering
Fluidics
amnis
Startup
Shutdown
Abort
Sheath
Flush
Compensation
Focus
Flow
ASSIST

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Autosampler option

The autosampler enables unattended operation of samples in 96 well plates loaded into the FlowSight. Prior to running the plate, a plate definition is created that assigns instrument settings to the wells, names to the output files, and parameters to include in a well plate report. While the plate is running, the user may be notified of any errors encountered via email. The instrument can also sterilize at the completion of the plate. This document describes the steps involved in operation the FlowSight autosampler.

Workflow:

1 Startup the instrument and calibrate as described under normal operations.

2 Create Instrument Settings Template(s) (.ist) to be used for each well on your plate. To do this, run an experimental sample manually with all of the fluorescence dyes to be used in the experiment (see INSPIRE Setup Quick Start Guide). Save each relevant template.

3 Create a Well Plate Definition (.def) that assigns instrument settings to wells, names to the sample output files, and parameters to include in the plate report (see procedure below).

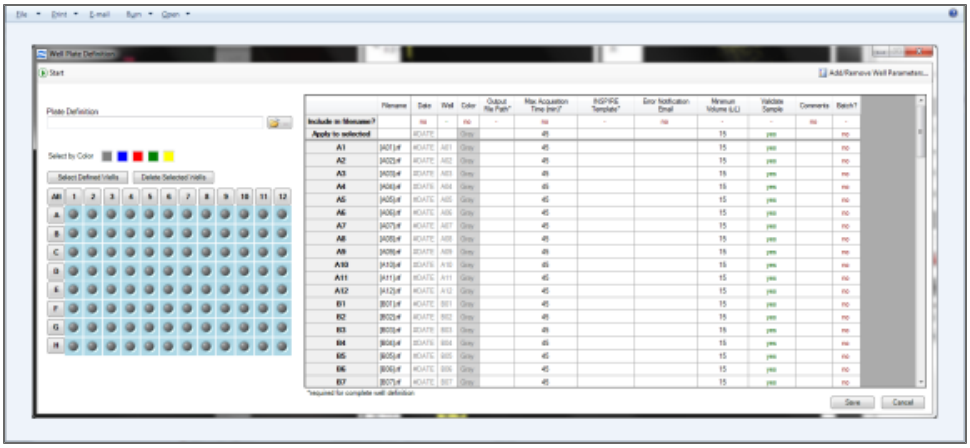
4 Add 75 υl samples to the wells of a u-bottom 96 well plate and cover with Sigma-Aldrich X-Pierce Film (XP-100, Cat # 2722502) and load the plate into the autosampler.

5 Run the plate (see procedure below).

Define the plate:

The steps required to create a Well Plate Definition (.def) and run a plate are outlined below:

1 Choose **Define Plate** from the Autosampler menu to open the Well Plate Definition window.



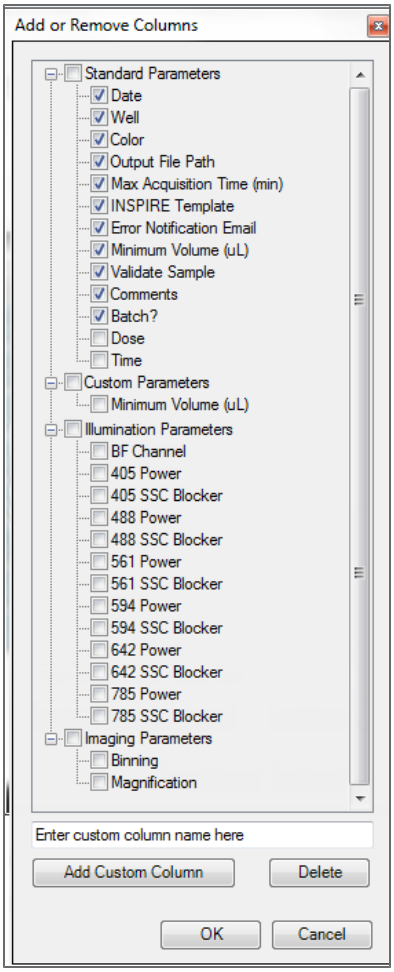
2 Browse for a previously saved definition (to edit) by clicking on the folder icon or continue to create a new definition.

3 **Name** the plate definition.

4 At a minimum, each well requires an Output File Path, Max Acquisition Time, and Template File in order to be considered 'defined'. Optional parameters can be added to the definition in the next step.
   - To include a parameter in the file name, click in the box below the column heading (make sure it says 'yes').
   - Columns can be re-ordered by click/drag.

5 Click **Add/Remove Well Parameters** to choose the parameters you want to report for the wells. There are several categories of parameters that may be chosen as a group or individually. Check or uncheck the desired parameters. The user can also define custom parameters. Expand the category to see the individual parameters. To delete a custom parameter, select it and use the delete key. Click **OK** when done.

6 Batching of the data into IDEAS may be done if a compensation matrix and template exists for the experiment.

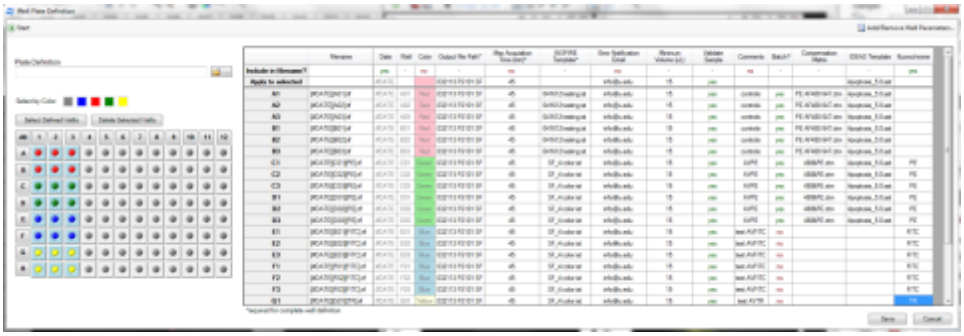


7 **Select** wells to define by clicking a) individually (or Ctrl click / shift click for multi-select), b) rows or columns, c) color, d) the 'Select Defined' button or e) All.

   Note: Selecting and defining wells with shared parametersfirst and then refining the definition for sets of wells makes it easier to organize the definition.

8 You can **edit values** for some of the Custom and many of the Standard parameters. You can do this for all selected wells or for individual wells. For example, if

you want to collect with Max Acquisition Time 10 minutes for the selected wells, type 10 in the 'Apply to selected' box below the Max Acquisition Time heading. If you want to only apply this to a single well, type this value in the box corresponding to that well. Below is an example of a Well Plate Definition using several parameters and showing only defined wells.

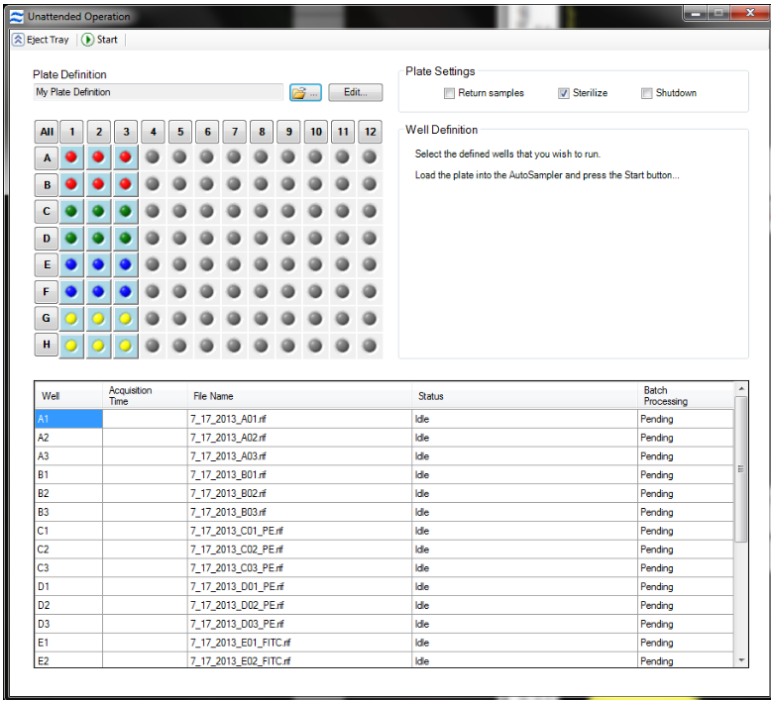


9 Highly recommended – select **Error notification Email** from the list of Standard parameters and type in the user email address in the 'Apply to Selected' box.

10 When done click **Save**.

Start the autosampler

11 Click **Start** to run the plate. The Auto Sampler Unattended Operation window opens with the Plate Definition you just saved. If you wish to choose a different Definition, browse for it by clicking on the folder icon. If you want to edit the Plate Definition, click Edit This Plate and you will be taken to the Well Plate Definition window.



12 Check or uncheck the boxes **Return samples, Sterilize, Shutdown**. Note that these boxes may be checked or unchecked while the plate is running. The operation will apply after the current sample is finished.

13 **Select** the wells to run (they will appear in the list).

Load the plate

14 Click **Eject Tray** to extend the plate nest.

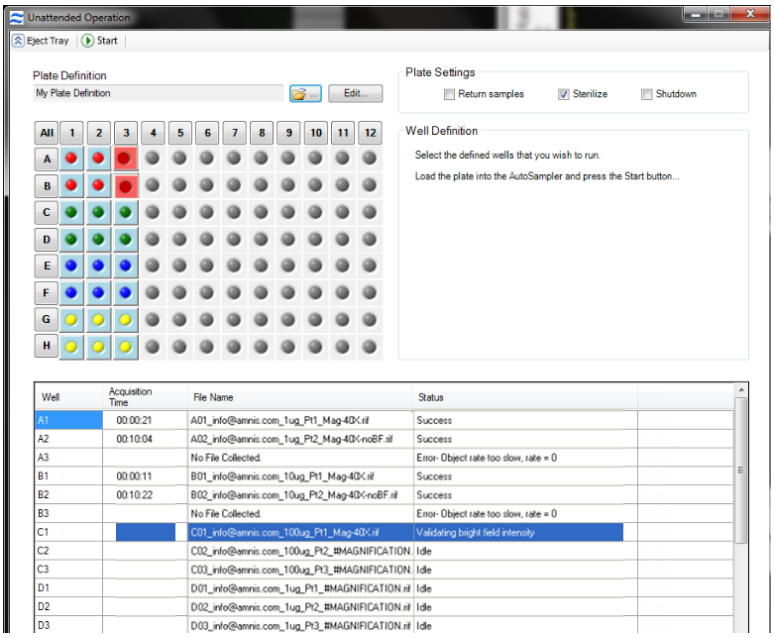
15 Add at a minimum 75 υl samples to the wells of a u-bottom 96 well plate and cover with Sigma-Aldrich X-Pierce Film (XP-100, Cat # 2722502) and load the plate into the autosampler.

16 Place your plate in the nest with well A1 positioned at the upper left corner.

17 Click **Start** to begin.

18 The Status column will be updated for each well as it is run. For each sample, the instrument loads the sample, performs validations, acquires the data and reports the result (success or error).

19 For example, here is a run with a failure to detect objects in column 3 for the first 2 rows (A3 and B3 failed). The wells are shown in red and the error status is displayed. An alert email was sent as entered in the plate definition.



During a run:

- You may stop the plate at any time by clicking the Stop button. This does not initiate sterilize (even if the Sterilize after running plate box is checked).
- Should the sheath tank or beads reservoir become empty or the waste tank full during a run, an alert will be sent to the email entered in the well plate definition. Acquisition will pause until the user intervenes.
- If an error occurs on a well, the sample is returned, an alert is sent to the email address entered in the well plate definition, and the autosampler moves on to the next well.
- If the same error occurs on three consecutive wells, the autosampler aborts the plate and sterilizes the instrument (if the 'Sterilize after running plate' box is checked)

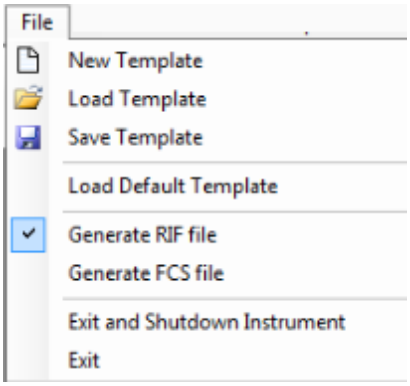
Report

20 A report will be saved (to the folder designated in the Output File Path of the plate definition) at the end of the run either when it was stopped manually or completed the entire plate.

Menus

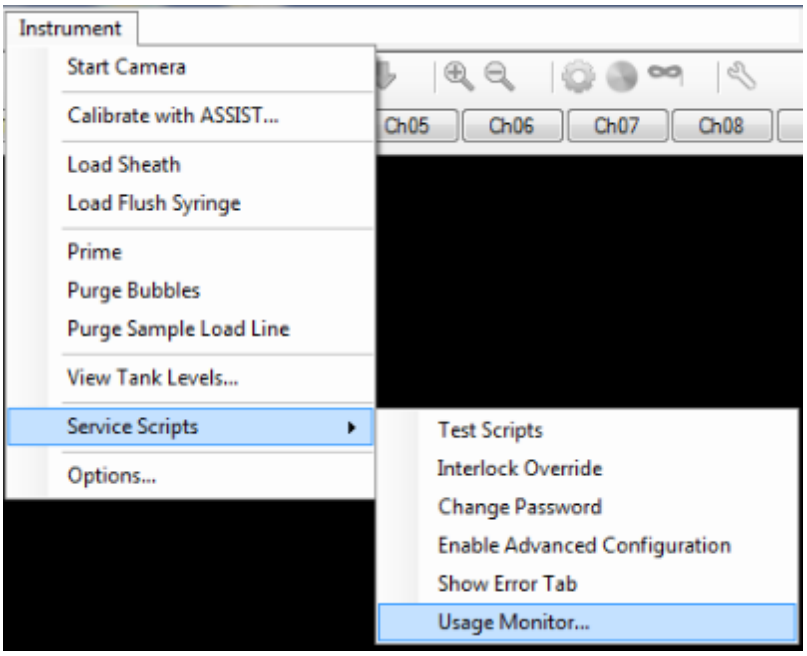
The menu bar is located in the upper-left portion of the INSPIRE screen. It consists of the following drop-down menus. This section describes the functions that are assessable from the menus

1 **File** menu: Load and save instrument setup templates. A template contains instrument settings that can be predefined and loaded to simplify the instrument setup process.



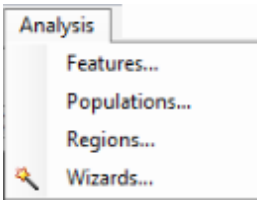
- **New Template:** Removes all graphs, populations and regions .
- **Load Template:** Browse for and open saved templates.
- **Save Template:** Save your settings as a template for future use. Template file names are appended with the suffix .ist. They are saved in the INSPIRE Data folder.
- **Load Default Template:** Loads factory settings.
- **Generate RIF file:** Check to save a Raw Image File during acquisition.
- **Generate FCS file:** Check to save a Flow Cytometry Standard file during acquisition.
- **Exit and Shutdown Instrument**: Turns off the instrument control system and exits INSPIRE. Note: this does not sterilize the system.
- **Exit**: Exits INSPIRE.
-

2 **Instrument**menu: Run the ImageStream^X camera and instrument-specific fluidic scripts (automated fluidic routines).



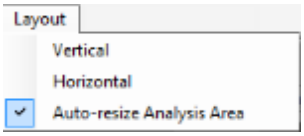
- **Start Camera**: Turns on imaging if it has stopped.
- **Calibrate with ASSIST:** Opens the Calibrations and Tests window.
- **Load Sheath:** Fills the sheath syringe with sheath fluid and an air bubble that facilitates stable flow.
- **Load Flush Syringe:** Fills the flush syringe with sheath fluid.
- **Prime:** Rapidly pushes a small volume of sample into the flow cell
- **Purge Bubbles**: Removes air bubbles from the flow cell by filling the flow cell with air then filling the sheath line and pump with debubbler and rinsing the flow cell. The sheath syringe is then refilled with sheath and the bubble trap, lines and flow cell are filled with sheath.
- **Purge Sample Load Line**: Cleans out the sample load line
- **View Tank Levels**: Opens the fluid level window.
- **Service Scripts**: For field service personnel only.- **Usage Monitor** is available to find the information on scripts that have run
- **Options**:

3 **Analysis** menu: Access the Feature, Population and Region Managers. Functionality is the same as for IDEAS. Refer to the IDEAS user manual for more information.



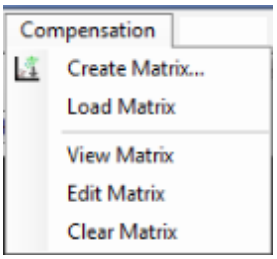
- **Features:** Opens the Feature Manager. Features can be renamed or new combined features can be created.
- **Populations:** Opens the Populations Manager. View,edit or delete populations.
- **Regions:** Opens the Regions Manager. View, edit or delete regions.
- **Wizards**: Opens the list of Wizards including the compensation wizard.

4 **Layout** menu:



- **Vertical:** View the image gallery and analysis area side by side.
- **Horizontal:** View the image gallery and analysis area top and bottom.
- **Auto-resize Analysis Area:** .Adjusts the layout to accomodate graphs and images when the image gallery changes size

5 **Compensation** menu: View, edit or create a new compensation matrix.



- **Create Matrix**: Starts the compensation wizard.
- **Load Matrix**: Apply an existing compensation matrix.
- **View matrix:** Opens the compensation matrix view.
- **Edit matrix:** Opens the compensation matrix for editing.
- **Clear matrix:** Stops applying a compensation matrix.

6 **Advanced:**menu: For field service personnel only.

7 **Help**: Access the current software version number.

- **About**: Software information including version number

- **Help**: Opens the user manul in a web browser.

Chapter 4: Troubleshooting

This section is designed to help you troubleshoot the operation of the FlowSight. If additional assistance is required, contact the Amnis service department.

System

Software

Image

Images appear pixelated or larger than normal

Not all 12 channels are being displayed

Images have incorrect colors

Intensity

Fluorescence imagery appears too dim

Fluorescence is too bright, images have a contrasting color or appear flat

One channel saturates while the others do not

Scatter is too dim or bright or changes over time

Large variation in brightfield intensity levels

Brightfield intensity level sets incorrectly

System

Symptom	Possible Causes	Recomended Solutions
Unstable fluidics (Air or clog in system)	Air bubbles in the sample	Make sure a sufficient sample volume is used, 50ul is recommended. To clear the air bubble: Run the purge bubbles script.
		Detergents and foaming agents (such as FBS) can cause bubbles to form in the lines. If these buffers are causing air in the system remove them from the sample and resuspend in dPBS. Run the purge bubbles script.
	Air bubbles in fluid lines	Run the sterilize script, followed by the startup script. Load calibration beads and verify the system runs normally.
	Clog in fluid lines	Filter the sample with a 70um nylon cell strainer. Run the sterilize script, followed by the startup script. Load calibration beads and verify the system runs normally.
	Sample is too concentrated	Clumpy and viscous samples cause cavitation in the fluidic lines and create bubbles. Dilute the sample to 1x10^7 cells/ml and strain the cells through a 70um nylon mesh. Run the purge bubbles script.

Symptom	Possible Causes	Recomended Solutions
	Inappropriate sheath solution	Verify the sheath is dPBS. De-gas the sheath as appropriate. Third party sheath buffers cannot be used on the FlowSight.
	Fluid lines are leaking	With the system powered down look for leaking sheath. Verify the fluid lines mount snuggly into position. Call Amnis service.
Fluidics respond sluggishly	Air buffer in the sheath syringe is not correct	The sheath syringe should contain 2-4 mL of air to buffer the movement of the pump's microstepper motor. If too little air is present run the "start-up" script.
	Fluid lines are leaking	With the system powered down look for leaking sheath. Verify the fluid lines mount snuggly into position. Call Amnis service.
Event rate slows over time	Cells have settled in the lines	Cells settle in the lines after 45-60 minutes of running, resulting in a drop in cell event rate. Stop and save the acquisition. Return the remaining sample, restore the sample volume to 30ul and re-load the sample to continue acquisition. Data can then be appended together in IDEAS.
	There is a clog or air bubble in the system	Run the purge bubbles script from the instrument drop-down menu. See solutions for unstable fluidics.
	Sample syringe is empty	Load a fresh sample
	Sheath syringe is empty	Load sheath, then go to the instrument drop down and run prime.
	Fluid lines are leaking	With the system powered down look for leaking sheath. Verify the fluid lines mount snuggly into position. Call Amnis service.
Event rate is slower than expected	Sample concentration is low	Make sure the sample concentration is between 10^7 and 10^8 cells/ml. Lower concentrations can be used but this will decrease the cells/second.
	Core is off center	Cropped images will be eliminated from data acquisition and if enough of the images are cropped the event rate can

Symptom	Possible Causes	Recomended Solutions
		appear lower than normal. Normally this is due to air in the system. Run the purge bubbles script from the instrument drop-down menu. See solutions for unstable fluidics.
	Insufficient illumination	Turn the appropriate lasers on. Set the 785 SSC laser to 40mw. Set the laser powers to maximum and decrease them to prevent pixel saturation.
Cross-contamination from previous samples	DNA dye from previous sample is labeling current sample	DNA dyes must be thoroughly flushed from the sample lines, to prevent residual dye from labeling subsequent samples. Load a sample of 10% bleach followed by a PBS wash, to remove all traces of the DNA dye in the instrument, or run the sterilize script (~30min).
	Cells from the previous sample are appearing in current sample	This suggests a minor clog. Load a sample of 10% bleach followed by a PBS wash to remove most contaminating cells, or run the sterilize script (~30min).
Erroneous fluid level indicator	Tank has moved away from the sensor	Open the buffer compartment and move the tank closer to the sensor until the fluid level indicator is correct.
	Sensor is broken	Power down and power up the instrument, if this does not fix the problem, call Amnis service.
Instrument will not pass ASSIST	Incorrect template loaded	Go to the file drop down and select “load default template”. Re-run ASSIST.
	Calibration particles are not running properly	The particles must be running >1,000 events per second, and without significant clumping. If the beads are diluted or clumped, try running a fresh tube of beads. If the problem persists there may be a fluidics issue, see the Flow rate stops or slows over time section.
	Calibration and/or test failure	Tests may fail if the system is reloading sheath, or failed to set up properly. Re-run the test by clicking in the box next to the test, and pressing the start button in

Symptom	Possible Causes	Recomended Solutions
		the popup window. If the test fails three times in a row, call Amnis service.
	Focus adjustor calibration failure	Verify brightfield is working properly.
	Frame Offset calibration failure	Verify brightfield is working properly.
	Spatial Offsets calibration failure	Verify brightfield is working properly.
	Dark Current calibration failure	Make sure the excitation lasers are off and brightfield is blocked. Completely power down the FlowSight and power back up to re-run the test.
	Brightfield XTalk calibration failure	Verify brightfield is working properly, and that spatial offsets passed.
	Horizontal Laser Calibration failure	Verify the laser turns on and can set power properly. Completely power down the FlowSight and power back up to re-run the test. Verify spatial offsets passed.
	Retro Calibration failure	Verify the laser turns on and can set power properly. Verify spatial offsets and frame offsets passed.
	Side Scatter Calibration failure	Verify the 785 SSC laser turns on and can set power properly. Completely power down the FlowSight and power back up to re-run the test. Verify spatial offsets passed.
	Laser Power test failure	Verify the laser turns on and can set power properly. Completely power down the FlowSight and power back up to re-run the test. Verify spatial offsets and frame offsets passed.
	Brightfield alignment test failure	Verify brightfield is working properly.
	Brightfield uniformity test failure	Verify brightfield is working properly.
	Camera noise test failure	Verify camera can image properly. Completely power down the FlowSight and power back up to re-run the test.
	Flow Core Axial Stability test failure	Verify the reagent buffers are full. Run the sterilize script followed by the startup

Symptom	Possible Causes	Recomended Solutions
		script, and re-run the test. See solutions for unstable fluidics.
	Flow Core Lateral Stability test failure	See solution above (Flow core Axial stability test).
	Flow Core Position test failure	See solution above (Flow core Axial stability test).
	Focus Percentage test failure	See solution above (Flow core Axial stability test).
	Focus Uniformity test failure	See solution above (Flow core Axial stability test).
	Image Quality test failure	See solution above (Flow core Axial stability test).
Compensation fails to compute values and save data	The region to collectis set incorrectly	In the Acquisition Settings section verify that 1,000 of “All” cells (or of a region drawn on the appropriate population) are being collected.
	Too many objects are being collected	Set the events to acquire less than 1,000.
	Cells are not fluorescent	Make sure that the compensation control sample has more than 10% positive events, and are as bright as possible. IgG capture beads or a cell line stained with a single fluorochrome may be used for comp controls.
	Cells are stained with more than one fluorochrome	Compensation controls must be a sample with a single fluorochrome label in a single tube. Each fluorochrome must be run separately.
	Cell concentration is too low when using the compensation wizard	Compensation controls must be running at 25 cells per second at a minimum

Software

Symptom	Possible Causes	Recomended Solutions
INSPIRE appears to freeze	Camera is not running	Click Run/Setup.

Symptom	Possible Causes	Recomended Solutions
	If the camera is already running	Click Stop then Run/Setup
	Imaging is paused	Click Resume.
	No objects in the current image view mode	In the cell view area, select the all population.
	A script is running	Wait until the script completes, or if necessary, click Abort Script to prematurely stop the operation.
	The INSPIRE application has crashed	Open the Windows Task Manager by simultaneously pressing <Ctrl + Alt + Del>. Click the Applications tab. If the INSPIRE for FlowSight status is 'Not Responding', select the INSPIRE task and click End Now. Restart the INSPIRE application by double clicking the FlowSight icon on the desktop. If the program restarts, make sure the lasers and brightfield lamp are turned on and then re-establish the core stream. If the application does not start, use the Windows Task Manager to end the INSPIRE task again. Shut the FlowSight computer down from the Start menu. Then turn on the instrument as described. If a crash occurs during the day, a complete shutdown is recommended at the end of the day, prior to running the sterilize script.
INSPIRE fails to launch	Splash screen is not responding	On the keyboard press Ctrl-Alt- delete, open the task manager, select INSPIRE for FlowSight and press end task. Wait 60 seconds and try restarting INSPIRE.
	Loss of communication between the computers and instrument.	Shut down the computer, and power off the instrument. Verify all computers are off. Power on the instrument and then the computer, wait 5 min and try launching INSPIRE.
Plots fail to update, or update slowly	Computer resources are being over used	Close all third party software.

Symptom	Possible Causes	Recomended Solutions
	Too many plots in the template	For optimal plot update rates limit the number of plots to 15.
	Parent population has no qualifying events	Right click on the plot, select graph properties, and change the selected population to “all” or a population that has qualifying events.
	Plots are scaled incorrectly	In the plot tool bar, press the – magnifying glass and rescale the plot.
Data file fails to collect	No events qualify for the region	Make sure there are events going into the collection region by viewing that region in the image gallery and updating the acquisition collection population appropriately.
		Verify the cell concentration is appropriate. 1x10^7 cells/ml is ideal.
	Computer hard drive is full	Verify the computer hard drive has sufficient room to save the data file. To do this go to Start / Computer / right click on properties and a pie chart showing how much disk space is available is displayed. Backup and delete data to free up disk space.
	Data file collected rapidly	Some samples have high concentrations and acquire faster than the display rate. Check the destination folder and see if the raw data was collected.
	File directory was lost	Collecting data over a downed network or changing the name of the destination folder will cause FlowSight to lose the data directory. Verify the data destination folder is accessible using the browse button in the Acquisition Settings section.
	No .rif or .fcs file was created	Go to the file drop down menu and check Generate .rif and or .fcs file.

Image

Symptom	Possible Causes	Recomended Solutions
No Images	Camera is not running	Click Run/Setup.
	If the camera is already running	Click Stop to stop the camera, and then click Run/Setup.
	Imaging is paused	Click Resume.
	Displayed region is incorrect	In the cell view area, select the all population.
	Insufficient illumination	Turn the appropriate lasers on. Set the 785 SSC laser to 40mw. Set the laser powers to maximum and decrease them to prevent pixel saturation.
		Make sure the brightfield lamp is turned on andclick Set Intensity.
	Core stream is outside the objective's field of view	Manually find the core stream. In the focus and centering section, move core track left or right to find the core.
	Computer resources are being over used	Close all third party software.
Imaging and acquisition rate is erratic, or appears frozen	Sample concentration is low	Make sure the sample concentration is between 107 and 108 cells/ml. Lower concentrations can be used but this will decrease the cells/second.
	Region being viewed has few or no cells	In the cell view area, select the all population, or readjust regions to include more cells.
	Insufficient illumination	Turn the appropriate lasers on. Set the 785 SSC laser to 40mw. Set the laser powers to maximum and decrease them to prevent pixel saturation.
		Make sure the brightfield lamp is turned on andclick Set Intensity.
	The sample is too concentrated	The process of object detection can safely handle up to 4000 objects per second. The maximum sample concentration is 4-5x108 cells per mL, with the recommended concentration 1-10 x107 cells per ml. To decrease the event rate, dilute the sample.
	The sample has an	Use a region to eliminate the debris

Symptom	Possible Causes	Recomended Solutions
	excessive amount of debris	from the data file, or prepare a fresh sample.
	Computer resources are being overused	Exit all third party programs.
Objects appear streaked	Camera is not tracking the cell velocity accurately	Verify brightfield is working normally and rerun ASSIST using calibration beads. See solutions for unstable fluidics.
Objects are not centered in the channel	Lateral deviation of the core stream due to air or clog in the system	Run the purge bubbles script from the instrument drop-down menu. See solutions for unstable fluidics.
	Autofocus and centering is not tracking properly	In the Focus and Centering section, adjust focus and centering left or right, until the images are centered and in optimal focus.
Objects are rotating in the core stream	Core stream position is grossly off-center within the flow cell due to air or clog in the fluidics	The core tracking and focus tracking should not change significantly from day to day. If either value changes radically, objects may rotate due to interactions with the sheath. An off-center core stream is caused by air or clogs in the fluidic system. Run the purge bubbles script from the instrument drop-down menu. See solutions for unstable fluidics.
Objects are out of focus	Camera line rate is incorrect	Re-run the focus adjuster and frame offset calibration in ASSIST, and verify it passes.
	Excessive core stream variation due to air or clog in the fluidics	Run the purge bubbles script from the instrument drop-down menu. See solutions for unstable fluidics.
	Core stream is moving too fast for the camera	Allow the system to settle for 60seconds after loading a sample. Collect data once imagery looks good.
	Autofocus is not tracking properly	In the Focus and Centering section, adjust focus and centering, left or right, until the images are centered and in optimal focus.

Symptom	Possible Causes	Recomended Solutions
Objects are cropped	Lateral deviation of the core stream due to air or clog in the system	Run the purge bubbles script from the instrument drop-down menu. See solutions for unstable fluidics.
	Autofocus and centering is not tracking properly	In the Focus and Centering section, adjust focus and centering left or right, until the images are centered and in optimal focus.
The two brightfield images are not of the same cell	Frame offset is incorrect	Run calibration particles on the Flow Sight. Load the default template and verify brightfield is in channel 1 and 9 at 800 counts of background. Open ASSIST re-run the frame offset calibration routine, and verify it passes.
	Illumination is grossly misaligned	Call service, and verify that the illumination pathways are in proper alignment.
	Cross correlation is incorrect	Run calibration particles on the Flow Sight. Load the default template and verify brightfield is in channel 1 and 9 at 800 counts of background. Open ASSIST, re-run the cross correlation utility, and verify it passes.
Images appear pixelated or larger than normal	Image gallery zoom is active	Use the – magnifying glass to zoom out and restore the native image size.
Not all 12 channels are being displayed	Image gallery zoom is active	Use the – magnifying glass to zoom out and restore the native image size.
	Channel is not activated	To activate a channel for acquisition, click on the channel column heading (i.e. Ch2) and check the “collected” check box to save that channel.
Images have incorrect colors	Image gallery display is set up incorrectly	Click on the channel column heading (i.e. Ch2) and set the gain and channel color.

Intensity

Symptom	Possible Causes	Recomended Solutions
Fluorescence imagery appears too dim	Image display settings are set too low	Increase the image display gain and/or change to log in the appropriate camera channel.
	Sample did not label well	Look at the sample with a fluorescent microscope.
	Insufficient illumination	Turn the appropriate lasers on. Set the laser powers to maximum and decrease them to prevent pixel saturation.
		If the probing protocol results in dim staining, sensitivity of the instrument can be increased by changing the fluidics speed to Lo / Hi sensitivity mode.
	Core stream position is grossly off-center within the flow cell due to air or clog in the fluidics	Run the purge bubbles script from the instrument drop-down menu. See solutions for unstable fluidics.
	Excitation laser is misaligned	Run calibration particles on the Flow Sight. Load the default template. Open ASSIST, re-run the laser alignment calibration for the appropriate laser line, and verify it passes.
Fluorescence is too bright, images have a contrasting color or appear flat	Image display settings are set too high	Decrease the image display gain and change to linear in the appropriate camera channel.
	Instrument sensitivity is set too high	Decrease the excitation laser power to prevent pixel saturation. Saturation is indicated in the image gallery by pixels colored in a contrasting color (generally red or white).
		Set the brightfield intensity to 800 counts by pressing “Set Intensity”.
	The sheath syringe is empty	Load sheath, then go to the instrument drop down and run prime.
	There is a clog or	Run the Purge Bubbles script from the

Symptom	Possible Causes	Recomended Solutions
	air bubble in the system	instrument drop-down menu. See solutions for unstable fluidics.
One channel saturates while the others do not	Instrument sensitivity is not optimized	The best instrument setup maximizes the dynamic range of fluorescence signal, while at the same time avoiding image pixel saturation (which cannot be compensated). In general decreasing the laser powers until no pixels saturate.
	Probing protocol requires better stain balance	Reduce the concentration of the stain that produces the saturating signal so that all probes can be simultaneously imaged without excessive saturation.
	Excessive fluorescent dye is left in the sample buffer.	Some DNA dyes are required to run with the sample to stain properly, however if too much dye is in solution it can cause the core stream to fluoresce. It's important to balance the concentration of these dyes so that the cells can be imaged properly. Typically the concentrations in "Current Protocols in Cytometry" should work.
Scatter is too dim or bright or changes over time	Instrument is experiencing large temperature variation	Allow the instrument to warm up by running for 15 minutes.
		Direct a fan toward the back of the instrument to dissipate excess heat, or move the system to a temperature controlled environment.
	Laser power set too high or low	Increase or decrease the 785 SSC laser power.
	Core stream position is grossly off-center within the flow cell due to air or clog in the fluidics	Run the purge bubbles script from the instrument drop-down menu. See solutions for unstable fluidics.
Large variation in brightfield intensity levels	Large flow speed variation due to air	Run the purge bubbles script from the instrument drop-down menu. See solutions for unstable fluidics.

Symptom	Possible Causes	Recomended Solutions
	Light source delivering variable output	Power down and power up the instrument, if this does not fix the problem, call Amnis service.
Brightfield intensity level sets incorrectly	Intensity set before desired flow speed has been achieved	Allow the system to stabilize after loading a sample, and then click Set Intensity.
	Movable optics are out of position	Power down and power up the instrument, if this does not fix the problem, call Amnis service.

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